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# THE JOURNAL

OF

# BIOLOGICAL CHEMISTRY

FOUNDED BY CHRISTIAN A. HERTER AND SUSTAINED IN PART BY THE CHRISTIAN A. HERTER  
MEMORIAL FUND

OFFICIAL ORGAN OF THE AMERICAN SOCIETY OF BIOLOGICAL CHEMISTS

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VOLUME XLVII

BALTIMORE

1921

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501  
517  
V. 47-48  
COPY

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THE JOURNAL OF BIOLOGICAL CHEMISTRY

PUBLISHED BY THE ROCKEFELLER INSTITUTE FOR MEDICAL RESEARCH FOR THE  
JOURNAL OF BIOLOGICAL CHEMISTRY, INC.

WAVERLY PRESS  
THE WILLIAMS & WILKINS COMPANY  
BALTIMORE, U. S. A.

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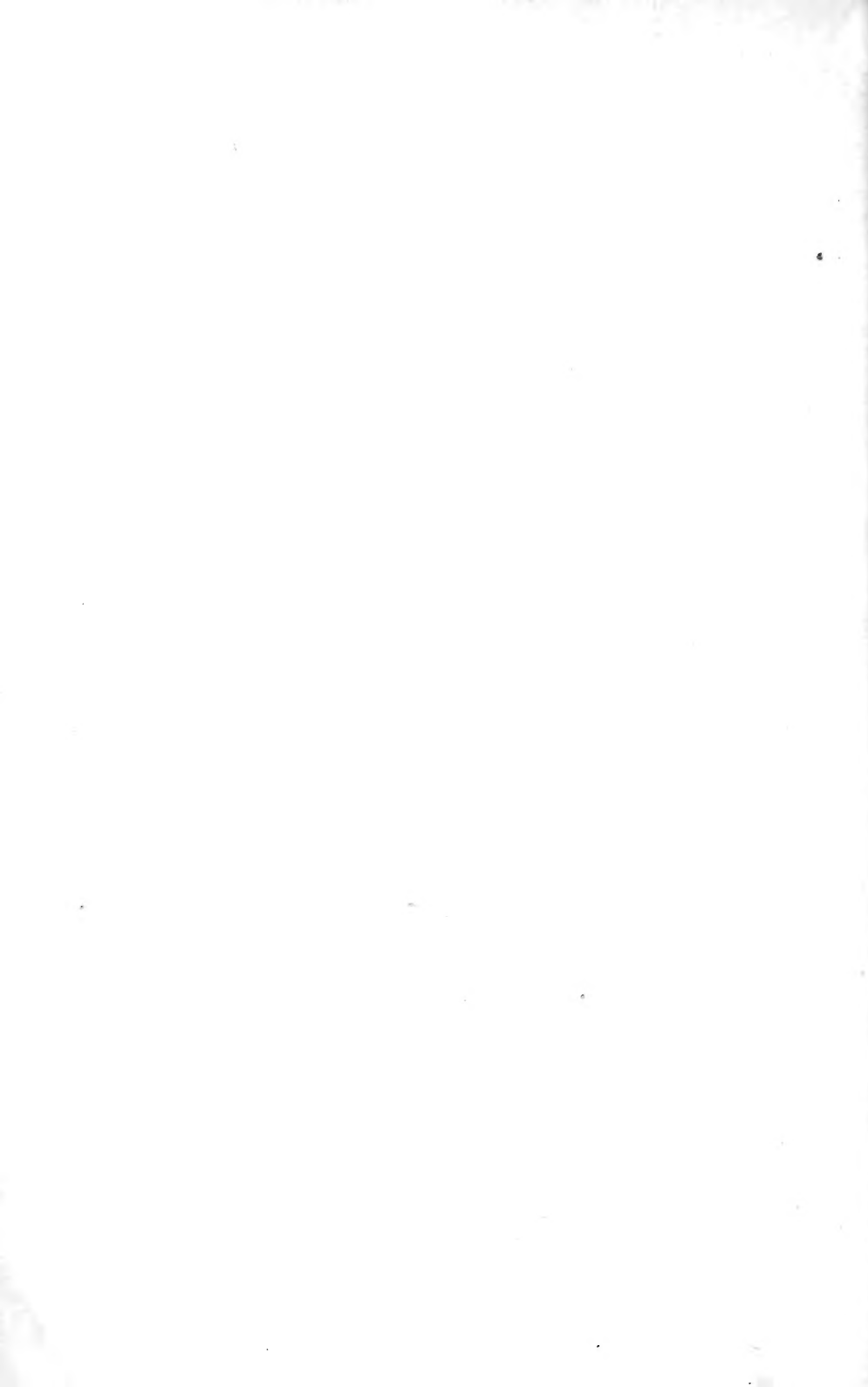
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## PREPARATION OF GALACTOSE.

By E. P. CLARK.

*(From the Polarimetry Section, United States Bureau of Standards,  
Washington.)*

(Received for publication, April 11, 1921.)

A number of methods for preparing galactose<sup>1</sup> have been reported, but partly because the details of procedure are not given with sufficient exactness, and partly because, in many cases, unnecessarily drastic measures have been recommended which add to the cost and reduce the yield, these methods are not satisfactory. Recently, an unpublished method, originated in the Bureau of Chemistry, has been used quite successfully by many workers. However, it is not generally known. The method which we have worked out in order to meet the demands made upon this Bureau has the advantages over the above mentioned methods of giving more uniform results, higher yield, and lower cost, and is reported here that it may be useful to those requiring a supply of this sugar.

1,500 gm. of lactose are dissolved in 3,750 cc. of hot water containing 75 gm. of concentrated sulfuric acid. The solution is brought to a boil and then simmered for 2 hours. A thin paste of barium carbonate is then added to the hot solution until it reacts neutral to Congo paper. The precipitate of barium sulfate is allowed to settle over night, after which as much as possible of the supernatant liquid is drawn off. This liquid is filtered through a thin layer of active carbon placed on moistened filter paper in a Buchner funnel. When all has passed through, the precipitate is placed on the filter, drained as dry as possible, and finally washed by drawing a little water through it. The filter, prepared with a small amount of carbon, as outlined, clarifies the solution and at the same time prevents the sulfate from passing through and gives a relatively rapid filtration.

<sup>1</sup> von Lippmann, E. O., *Die Chemie der Zuckerarten*, 1904, iii, 698-700.

The filtrate is concentrated under diminished pressure until it has a weight of 1,650 gm. If a refractometer is available, the concentrated syrup should have a refractive index between 1.5120 and 1.5125. The very thick syrup is warmed to between 60 and 70° C. and 250 cc. of ethyl alcohol are dissolved in it by vigorous shaking. The solution is then poured into a beaker or jar and the remaining syrup is washed from the flask with 500 cc. of methyl alcohol. This is done best by adding the methyl alcohol to the flask portionwise, warming, and shaking in a water bath. The whole solution is thoroughly mixed, seeded with some pure galactose crystals, and allowed to crystallize.

The crystallization is generally complete in about 4 days, after which the galactose is filtered off, washed with a little methyl alcohol, then with 85 per cent ethyl alcohol, and finally with 95 per cent alcohol. It is then dried. The yield of this crude sugar is about 27 per cent of the lactose taken.

In order to purify the crude galactose, it is dissolved in water, making about a 25 per cent solution. To this is added a few cc. of glacial acetic acid. It is then concentrated under diminished pressure to about 75 per cent total solids, warmed to 60 or 70° C., transferred to a beaker, and 95 per cent alcohol added to saturation. Almost immediately the contents of the flask become solid. After allowing to stand over night, it is filtered from the mother liquor, washed, and dried. The yield is generally 82 to 83 per cent of the crude sugar taken.

A thoroughly dried sample of this recrystallized product had the following properties:

Ash	=	0.034 per cent.
$[\alpha]_D^{25}$	=	80.4° in 10.617 per cent aqueous solution.
$[\alpha]_{\lambda 5461 \text{ Å. u.}}^{20}$	=	95.8° " 10.617 " " " "

The values are only given, however, to identify the product. Work upon the thorough purification and the accurate determination of its specific rotation is in progress.

# A NOTE ON THE ABSORPTION OF CALCIUM SALTS IN MAN.

BY EDWARD H. MASON.

(From the Metabolism Clinic, the Royal Victoria Hospital, Montreal, Canada.)

(Received for publication, April 7, 1921.)

Until recently, calcium salts, especially the lactate, have been extensively employed as hemostatics. Their use has been largely empirical for there is little experimental evidence to show that the

TABLE I.

Case.	Dose.	Plasma calcium (as Ca) per 100 cc.				Maximum increase.
		Before.	1 hr.	2 hrs.	3 hrs.	
		mg.	mg.	mg.	mg.	
1	Calcium lactate 5 gm.....	10.2	12.8	11.2	13.0	+2.6
2	" " 5 " .....	13.0	13.0	13.0	13.0	0
3	" " 5 " .....	12.0	11.7	12.0	12.2	0
1	Calcium lactate 5 gm. + 25 cc. cod liver oil.....	10.4	10.0	10.0	9.8	0
2	" " " .....	13.4	14.8	14.0	14.0	+1.4
3	" " " .....	13.0	14.6	12.8	13.1	+1.6
4	Calcium chloride 5 gm.....	14.8	16.2	16.0	15.2	+1.4
5	" " 5 " .....	11.2	13.2	13.8	Lost.	+2.6
6	" " 5 " .....	11.0	15.4	14.8	14.8	+4.4
7	" " 5 " .....	12.6	15.0	15.2	15.2	+2.6
8	Calcium lactate 5 gm. + 25 cc. 0.05 N HCl.....	11.4	11.2	9.8	9.4	0
9	" " " .....	8.4	10.4	9.6	9.6	+2.0
10	Calcium chloride 5 gm. + 25 cc. 0.05 N HCl.....	11.2	13.2	13.2	13.6	+2.4
11	" " " .....	11.6	12.4	12.8	12.8	+1.2

calcium content of the blood can be influenced by their oral administration when its calcium concentration is already normal.

Halverson, Mohler, and Bergeim (1) showed that in certain cases of uremia with a low blood calcium the latter could be ap-

preciably affected by the oral administration of calcium lactate. Denis and Minot (2) also agreed that it is usually impossible to influence the calcium content of the plasma by calcium lactate ingestion.

A short series of observations has been completed on normal subjects. The determinations were made by Lyman's (3) technique, using citrated plasma as advocated by Halverson, Mohler, and Bergeim. Two salts, the lactate and the chloride, were used. Both were administered in 5 gm. doses in 300 cc. of water on a fasting stomach. The plasma values before, and each hour afterwards for 3 hours, were determined. Likewise, both salts were dissolved in 25 cc. of 0.05 N HCl, and given with 250 cc. of water. Also the lactate was given with 25 cc. of cod liver oil.

The results are shown in Table I.

From the results in Table I it would appear that a single large dose of calcium lactate given in either of the above three forms little influences the plasma values. Calcium chloride seems to be absorbed better and shows more consistent increases in the plasma values. Its solution in weak hydrochloric acid does not affect its rate of absorption.

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1. Halverson, J. O., Mohler, H. K., and Bergeim, O., *J. Biol. Chem.*, 1917, xxxii, 171.
2. Denis, W., and Minot, A. S., *J. Biol. Chem.*, 1920, xli, 357.
3. Lyman, H., *J. Biol. Chem.*, 1917, xxix, 169.

# DINITROSALICYLIC ACID: A REAGENT FOR THE ESTIMATION OF SUGAR IN NORMAL AND DIABETIC URINE.

BY JAMES B. SUMNER.

WITH THE ASSISTANCE OF V. A. GRAHAM.

(From the Departments of Physiology and Biochemistry, Medical College, Cornell University, Ithaca.)

(Received for publication, March 26, 1921.)

The methods used for the quantitative determination of sugar in urine, including the polariscope, give good results when the amount of sugar present is considerable, but there is always a point where the urine contains so little sugar that the results are dubious. Special methods have been published for the determination of sugar in normal urine. Many of these give results that are too high, and others, like the method of Benedict and Osterberg,<sup>1</sup> are too long for clinical work.

It appeared that some reagent might be found which could be used both qualitatively and quantitatively and which could be applied to both normal and diabetic urine. The author believed that the most suitable substance would be a compound similar to picric acid in that it would be reduced by glucose to form a highly colored nitroamino compound, but dissimilar to picric acid in that it would neither form colored compounds with acetone or creatinine nor be reduced by urinary constituents other than the sugars.

Two nitro compounds have been found which meet most of these requirements; these are 4, 6-dinitroguaiacol and 3, 5-dinitrosalicylic acid.<sup>2</sup> Both of these compounds can be used successfully for the estimation of glucose in diabetic urine, but while they are not reduced in the presence of sodium carbonate by any urinary constituent yet tried, with the exception of the

<sup>1</sup> Benedict, S. R., and Osterberg, E., *J. Biol. Chem.*, 1918, xxxiv, 195.

<sup>2</sup> Hübner, H., *Ann. Chem.*, 1879, cxev, 45.

reducing sugars, they are reduced by such substances as uric acid and polyphenols when glucose is also present. Moreover, they are reduced by the urine as a whole even after the sugar present has been destroyed. The results obtained by the use of these reagents for the sugar in normal urine may be as much as 100 per cent too high, or even more in some cases.

The author with Miss V. A. Graham recently read a paper on the use of dinitroguaiacol at the meeting of the American Society of Biological Chemists and will not describe the use of dinitroguaiacol in this paper because he believes dinitrosalicylic acid is a far better reagent. While both compounds, under the same conditions of heating and alkalinity, give approximately the same results with normal urine, dinitrosalicylic acid possesses the advantages of being more soluble in the form of its sodium salt, of being lighter in color, and cheaper to prepare. In addition dinitrosalicylic acid is an excellent protein precipitant and on this account can probably be used for the determination of blood sugar.

Using dinitrosalicylic acid as a reagent, the author has found a way to obtain the true values for the sugar in normal urine as follows: 1 cc. of urine is heated in boiling water with 1 cc. of 3 per cent sodium hydroxide for 15 minutes. This treatment destroys the reducing sugars completely, provided the amount present is less than 1 mg. Either 0.5 or 1 mg. of glucose, depending upon the quantity of glucose estimated to have been present originally, is now added to the cooled solution, after which 1 cc. of dinitrosalicylate solution is added and the test-tube heated for 5 minutes. Any reduction exceeding that given by the added glucose is caused by substances which are not sugars. The reducing value of these substances is subtracted from the total reducing value of the urine, giving the value for sugar by difference. It has been found that the quantity of glucose added must be approximately equal to the amount destroyed by heating with alkali, otherwise an error is introduced.

That glucose when present in small amounts can be completely destroyed by heating with alkali has been carefully proved, using the sensitive copper and phosphomolybdic reagents used by Folin and Wu<sup>3</sup> for the determination of blood sugar. With

<sup>3</sup> Folin, O., and Wu, H., *J. Biol. Chem.*, 1919, xxxviii, 81.

these reagents it was found that heating 1 cc. of a solution of 1 mg. of glucose with 1 cc. of 3 per cent sodium hydroxide solution for 15 minutes destroyed all but 2 to 3 per cent of the sugar. Heating for 20 minutes destroyed all of the sugar. In practice the author heats the urine with alkali for 15 minutes and uses diluted urine when the reduction due to both sugar and other reducing substances is equivalent to more than 1.5 mg. The reduction due to sugar alone is usually about 60 per cent of the total reduction.

That the material in normal urine destroyed by heating with alkali is mostly sugar has been proved by fermenting urine with yeast. The yeast treatment is as follows: 50 cc. of urine together with a little paraffin and a few glass beads are weighed in a 100 cc. Erlenmeyer flask, acidified with a drop or two of glacial acetic acid if not already acid, and boiled for 5 minutes to remove dissolved toluene. The flask is cooled and water is added to restore the original weight after which the contents are well mixed with half a cake of Fleischmann's compressed yeast. The flask is stoppered and kept in an incubator at 32-33°C. for about 20 hours. Blanks containing pure glucose were fermented occasionally. It was found that with a glucose concentration of 1 mg. per cc. all the glucose was removed.

Table I shows the reduction given by unfermented urine before and after heating with alkali and by fermented urine before and after heating with alkali. A correction has been made for the water content of one-half a cake of yeast. This was found to amount to about 4.5 gm.

These results show plainly that normal urine contains something that reduces dinitrosalicylic acid, that is readily destroyed by heating with sodium hydroxide, and that is largely fermented by yeast. The results show also that the reducing material in normal urine which is not destroyed by heating with alkali is not fermented by yeast.

Shaffer and Hartmann<sup>4</sup> have recently published a paper in which they claim that normal urine contains little or no fermentable sugar. We are obliged to disagree with this statement.

*Preparation of 3, 5-Dinitrosalicylic Acid (the Author's Method).—*Mix 75 gm. of concentrated sulfuric acid with 15 gm. of concen-

<sup>4</sup> Shaffer, P. A., and Hartmann, A. F., *J. Biol. Chem.*, 1920-21, xlv, 365.

trated nitric acid and cool in ice water. When well cooled add with shaking 15 gm. of salicylic acid in small portions at a time, keeping the preparation well cooled. When all the salicylic acid has been added the material is poured into about 800 cc. of water, cooled for some time, and filtered off by suction. The crystals of dinitrosalicylic acid are dissolved by heating in dilute sodium carbonate solution, filtered, and salted out by adding a large excess of saturated sodium carbonate solution, followed by cool-

TABLE I.  
*Percentage Reduction of Normal Urine.*

Urine No.	Before fermentation.		After fermentation.	
	Total reduction.	After heating with alkali.	Total reduction.	After heating with alkali.
1	0.041	0.022	0.022	0.025
2	0.106	0.061	0.065	0.061
3	0.074	0.032	0.034	0.032
4	0.088	0.031	0.044	0.029
5	0.126	0.050	0.061	0.050
6	0.165	0.065	0.087	0.057
7	0.120	0.046	0.053	0.055
8	0.090	0.040	0.053	0.042
9	0.232	0.083	0.123	0.080
10	0.087	0.033	0.034	0.033
Average....	0.113	0.046	0.058	0.046

Total sugar..... 0.067 (0.113-0.046)

Fermentable sugar..... 0.055 (0.113-0.058)

Unfermentable sugar..... 0.012 (0.058-0.046)

ing. The sodium salt is filtered off by suction and washed with saturated sodium carbonate solution. The material is now dissolved in hot water, filtered, and precipitated as the free acid by adding a large excess of strong hydrochloric acid. After cooling, the dinitrosalicylic acid is filtered off, pressed, recrystallized once from a small amount of boiling water, and dried to constant weight at 100°C.

*Preparation of Dinitrosalicylate Solution.*—Dissolve 2 gm. of dinitrosalicylic acid in about 70 cc. of hot water with the aid of 10 cc. of 20 per cent sodium carbonate solution. The solution is cooled and made up to 100 cc. volume.



*Sodium Hydroxide Solutions.*—Two solutions of sodium hydroxide are required, containing 1.5 and 3.0 per cent of sodium hydroxide, respectively.

*Glucose Solutions.*—Solutions containing 1 and 0.5 mg. of glucose per cc. are prepared and preserved with toluene.

*Method.*

Pipette 1 cc. of urine into a 1.5 by 15 cm. test-tube; add 1 cc. of the 2 per cent dinitrosalicylate solution and 2 cc. of the 1.5 per cent sodium hydroxide solution. Mix, plug with cotton, and heat in boiling water for 5 minutes. Cool and dilute to 25, 50, or 100 cc. volume according to the depth of color. Mix and compare in colorimeter against standard. If the sugar content of the urine is greater than 0.4 per cent the test must be repeated with diluted urine. If the unknown solution contains an appreciable quantity of precipitate it should be centrifuged.

The above procedure gives with normal urine results that are too high because of the reducing action of uric acid and polyphenols. The reduction due to these substances is determined as follows: Pipette into a test-tube 1 cc. of urine and 1 cc. of 3 per cent sodium hydroxide solution. Mix, plug with cotton, and heat in boiling water for 15 minutes. If the total reduction has amounted to more than 1.5 mg. per cc. calculated as glucose, diluted urine must be used. After heating for 15 minutes cool and add 1 cc. of a solution containing either 1 or 0.5 mg. of glucose, according to whether the total reduction of the urine was more or less than 1 mg. per cc. calculated as glucose. Add 1 cc. of 2 per cent dinitrosalicylate solution, mix, plug with cotton, and heat for 5 minutes in boiling water, proceeding as above. After subtracting from the result obtained the amount of glucose added the remainder will be the value for the uric acid and polyphenols. This last is subtracted from the original reducing value of the urine and gives as the difference the value of the sugars. With diabetic urine this procedure is not necessary unless the sugar content is low.

*Standard.*—Heat 1 cc. of a solution containing 1 mg. of glucose with 1 cc. of the dinitrosalicylate solution and 2 cc. of the 1.5 per cent sodium hydroxide solution for 5 minutes; cool, dilute to 25 cc. volume, and mix. This standard will keep for several hours.



## NOTE ON THE GASOMETRIC DETERMINATION OF NITROGEN.

By RAYMOND L. STEHLE.

*(From the Laboratory of Physiological Chemistry, School of Medicine,  
University of Pennsylvania, Philadelphia.)*

(Received for publication, April 28, 1921.)

In a recent communication on this subject<sup>1</sup> it was observed that oxygen was liberated to some extent along with nitrogen when the Kjeldahl digest was treated with sodium hypobromite. The procedure there described called for its absorption with alkaline pyrogallate before reading the gas volume. An investigation as to the cause of the liberation of oxygen has shown that the copper sulfate added to hasten the digestion is responsible. Hence, if this substance is omitted, nitrogen only is liberated and there is no need for the pyrogallate treatment. Besides simplifying the manipulation, the gas volume can then be read with more accuracy, due to the fact that one is dealing with a water-clear solution instead of a highly colored one.

<sup>1</sup> Stehle, R. L., *J. Biol. Chem.*, 1920-21, xlv, 223.



## THE GASOMETRIC DETERMINATION OF UREA IN URINE.

By RAYMOND L. STEHLE.

(From the Laboratory of Physiological Chemistry, School of Medicine,  
University of Pennsylvania.)

(Received for publication, April 29, 1921.)

Several months ago<sup>1</sup> in a communication describing a gasometric method for the determination of total nitrogen, mention was made of the fact that when carried out *in vacuo* the reaction between sodium hypobromite and urea results in the liberation of the theoretical volume of nitrogen. The necessary vacuum is readily obtainable with the Van Slyke apparatus for determining the carbon dioxide content of blood plasma. Some effort was spent at the time in attempting to utilize the reaction for a blood urea method but it became evident that the error introduced by other nitrogenous constituents of blood was greater than permissible.

Recently Youngburg<sup>2</sup> has shown that the urease method for urinary urea may be somewhat simplified by first removing the ammonium salts and then carrying out the urease decomposition and aeration in the manner described by Van Slyke and Cullen.<sup>3</sup> The ammonium salts are removed by shaking the urine with permutit. The idea immediately suggested itself that here was a place to apply the hypobromite reaction. In the older hypobromite methods the results were unsatisfactory because the reaction (using pure urea solutions) was found to give less than the theoretical amount of nitrogen even when allowed to continue for hours, and in addition ammonium salts and other urinary constituents also yielded nitrogen when subjected to the action of hypobromite. With the aid of permutit the ammonium salts

<sup>1</sup> Stehle, R. L., *J. Biol. Chem.*, 1920-21, xlv, 223.

<sup>2</sup> Youngburg, G. E., *J. Biol. Chem.*, 1920-21, xlv, 391.

<sup>3</sup> Van Slyke, D. D., and Cullen, G. E., *J. Biol. Chem.*, 1914, xix, 211.

may now be eliminated as sources of error. It remained, therefore, to determine the error which might be introduced by other substances present in urine.

Assuming for the moment that an average 24 hour specimen of urine contains 15 gm. of urea nitrogen, 0.300 gm. of creatinine nitrogen, and 0.250 gm. uric acid nitrogen, it is evident that if all the nitrogen of the two latter constituents was liberated quantitatively the error introduced would be about 3.7 per cent. In order to determine the actual quantities liberated these two compounds were subjected to the action of hypobromite. It was found that creatinine yielded about one-seventh of its ni-

TABLE I.  
*Urea Nitrogen, per 1 Cc. of Urine.*

Hypobromite.		Van Slyke and Cullen.		Youngburg.	
<i>mg.</i>		<i>mg.</i>		<i>mg.</i>	
7.60	7.48	7.53	7.53	7.50	7.62
7.52	7.55	7.75	7.75	7.56	7.56
6.43	6.43	6.46	6.40	6.44	
5.16		5.12	5.18	5.26	
6.89	6.89	6.83	6.89	6.78	6.88
6.66	6.71	6.67	6.73	6.60	6.72
4.72	4.72	4.61	4.72	4.70	4.87
14.4*	14.5	14.2	14.2	14.3	14.6
11.0*		10.9	10.9	10.8	10.9

\* Dog urine.

trogen while uric acid yielded about one-half. In the case of the latter, however, the evolution of nitrogen takes place slowly and in consequence, by limiting the reaction time to that required for the urea reaction to go to completion, the error need not exceed about 0.3 per cent.

Conceivably, hippuric acid and amino-acids might be sources of error. However, the first does not yield any nitrogen with hypobromite. Glycocoll yields about 3 per cent of its N. Inasmuch as the amount of amino-acids present in urine is very small to begin with<sup>4</sup> and provided that other amino-acids conduct themselves similarly to glycocoll, it is evident that the error from this direction is negligible. Creatine, which is

<sup>4</sup> Levene, P. A., and Van Slyke, D. D., *J. Biol. Chem.*, 1912, xii, 301.

sometimes a constituent of urine, liberates 2 of its 3 nitrogen atoms under the conditions of the experiment. Since it is usually absent or present only in small amounts this source of nitrogen may in general be neglected. Allantoin conducts itself very similarly to uric acid.

Table I contains some results obtained by the hypobromite method, and the urease method. In the case of the latter, determinations were made by Youngburg's modification as well as by the usual procedure of Van Slyke and Cullen.

In view of the general satisfaction given by the urease method the present method may seem superfluous. It is fitting, therefore, that its advantages be stated. They are: (1) Rapidity. Starting with a sample of urine the urea content may be known in 10 minutes. (2) Standard solutions are not employed. (3) Reagents necessary are simple and easily prepared. (4) There is practically no opportunity in the procedure for things to go awry as there are opportunities in the urease method. For example, the keeping qualities of dilute standard solutions and of urease solutions are not matters of concern. Neither is there any question of foaming nor about how long and at what rate to aerate.

#### *Procedure.*

25 cc. of diluted urine (diluted in the ratio of 1:10) are shaken with 4 gm. of permutit for 4 minutes. The mixture is then centrifuged or filtered. 1 cc. of the  $\text{NH}_3$ -free urine is introduced into the Van Slyke  $\text{CO}_2$  apparatus, the last portion being rinsed in with 1 cc. of water followed by 1 cc. of sodium hypobromite solution. (This is made by mixing equal volumes of two solutions, one containing 12.5 gm. of sodium bromide and 12.5 gm. of bromide per 100 cc. and the other 28 gm. of sodium hydroxide per 100 cc.) The mercury is lowered to the 50 cc. mark and the apparatus is then shaken vigorously for about half a minute. The aqueous solution is collected in the proper chamber below the lower stop-cock, mercury is admitted to the 50 cc. chamber, and after adjusting the pressure the volume of nitrogen is read. Correction is then made for the dissolved air con-

tained in the diluted urine, the rinse water, and the hypobromite solution.<sup>5</sup>

It may be assumed with reasonable accuracy that the solubility of air in the diluted urine is the same as in pure water. For temperatures between 15 and 30°C. and a pressure of 1 atmosphere the solubilities are as given in Table II. The volumes are those which the gas would occupy at 760 mm. and the temperature in question and may, therefore, be subtracted from the gas volume as read. Determinations of the air dissolved in the hypobromite solution showed that between 15 and 20°C. this amounts to 0.006 cc. and between 21 and 25° to 0.005 cc.

TABLE II.

*Cc. of Air Measured at 760 mm. of Mercury and the Temperature in Question per 1 Cc. of Water.*

Temperature.	Volume.	Temperature.	Volume.	Temperature.	Volume.
°C.	cc.	°C.	cc.	°C.	cc.
15	0.0216	21	0.0198	27	0.0184
16	0.0212	22	0.0196	28	0.0182
17	0.0209	23	0.0193	29	0.0180
18	0.0206	24	0.0191	30	0.0178
19	0.0203	25	0.0188		
20	0.0201	26	0.0186		

The corrected volume is then reduced to standard conditions (0° and 760 mm. mercury) by means of the following formula.

$$V_o = V \frac{P_o - h}{(1 + 0.00367 t) 760}$$

where

$V$  = gas volume as measured.

$P_o$  = corrected barometric pressure.

$h$  = aqueous tension.

$t$  = temperature at which gas was measured.

To facilitate the calculation it will be found advantageous to refer to almost any of the compilations of physical and chemical data for useful gas reduction tables.

Once the volume of nitrogen measured at 0° and 760 mm. of Hg has been determined the weight is determined by mul-

<sup>5</sup> The correction for the air content of the diluted urine and rinse water can be eliminated by extracting the two in the apparatus and expelling the extracted gases before adding the hypobromite.



tiplying by 0.0012507, the weight of 1 cc. of nitrogen. Taking the dilution and amount of sample into consideration the amount of urea nitrogen per 1 cc. of urine is easily found.

Some consideration was given to the adaptability of the hypobromite reaction to the determination of ammonium salts in urine as well as to the determination of urea. Theoretically there are no difficulties. The difference between the quantities of nitrogen liberated by diluted whole urine and by urine treated with permutit represents ammonia N and that only. The difficulty is in the measurement of the two quantities with sufficient accuracy to make it possible to express the ammonia N concentration with at least two significant figures. The ordinary Van Slyke apparatus cannot be read without a possible error of at least 0.003 of a cc. in the opinion of the writer. In addition no protection is provided against slight differences between the temperatures of the gas itself and the air registered by the thermometer close by. Consequently since the ammonia N is usually less than one-tenth of the sum of the ammonia and urea nitrogen and therefore occupies less than one-tenth of a cc. in the apparatus, its volume cannot be expressed accurately in thousandths of a cc. as is necessary if the result is to be expressible with two significant figures. If an approximate relation between the urea N and ammonia N is desired this can be very readily obtained.

#### SUMMARY.

A method for determining urea in urine is described which is both brief and accurate. Ammonium salts are removed by treating the urine with permutit and the ammonium-free solution is then subjected to the action of sodium hypobromite in the vacuum obtained with a Van Slyke CO<sub>2</sub> apparatus. Nitrogen is liberated quantitatively from the urea but to an entirely negligible extent from other urinary constituents.

Comparative analyses obtained with the urease and hypobromite procedures demonstrate the accuracy of the method.



## FURTHER IMPROVEMENTS IN THE NEPHELOMETER-COLORIMETER.

BY PHILIP ADOLPH KOBER AND ROBERT E. KLETT.

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(Received for publication, March 29, 1921.)

The colorimeter described by Kober<sup>1</sup> has been improved in certain details which have resulted in the instrument described in the present paper. The first departure in construction from the Duboseq model was made by Kober<sup>1</sup> in 1915. It consisted of a 100 mm. scale, tiltable stand, adjustable verniers, fused black glass plungers, a rack, and a pinion. In 1917 this model was abandoned for the present one,<sup>2</sup> which has incorporated in it a fine screw arrangement in back of the instrument, away from possible contact with corroding liquids overflowing from the cups, in place of the rack and pinion exposed to such accidental overflowing by virtue of its position, retaining the fused black glass plungers with clear glass bottoms, but also having fused cups made of different colored glass which, in conjunction with the black plungers, made the transformation of the colorimeter into a nephelometer a matter of simply changing the cups. This 1917 model also possessed an ingenious device for adjusting the verniers to the zero point. Besides these changes there was added a set of split reflectors for the regulation and adjustment of light reflection. The hollow black plungers having fused optical ends, have been found to possess a special advantage in hydrogen ion determinations by Duggar.<sup>3</sup> He found that by putting shield solutions into the plungers as well as into the cups, a comparator for hydrogen ion and other work of great accuracy resulted. The split reflectors have also been found of special use by Field.<sup>4</sup> Where an unknown solution has a slight turbidity which absorbs light, thereby darkening the transmitted light, Field operates the reflector underneath the standard solution so that equal darkening is obtained.

Bock and Benedict<sup>5</sup> in 1918 described an instrument that is in reality a half plunger and a half instrument. This instrument has one advantage over most instruments of the plunger type; namely, that the scale

<sup>1</sup> Kober, P. A., and Graves, S. S., *J. Ind. and Eng. Chem.*, 1915, vii, 843.

<sup>2</sup> Kober, P. A., *J. Biol. Chem.*, 1917, xxix, 155.

<sup>3</sup> Duggar, B. M., *Annals of the Missouri Botanical Gardens*, 1919, vi, 179.

<sup>4</sup> Field, C. W., personal communication.

<sup>5</sup> Bock, J. C., and Benedict, S. R., *J. Biol. Chem.*, 1918, xxxv, 227.

and eyepiece can be viewed from one position of the observer, usually while sitting. The disadvantage of this type of instrument is the lack of symmetry or lack of interchangeability of the two sides of the instrument and of the two paths of light. Where only one or two heights of standard solution are necessary as in certain routine work this instrument is suitable, but this lack of flexibility in height of standard solution and the limited scale (40 mm.) make it less suitable for research and scientific work.

In 1918-19 Leitz<sup>6</sup> made a decided change in construction in one of their colorimeters, by substituting a lever arrangement for the rack and pinion. This departure from the rack and pinion (Duboseq) or from the fine screw arrangement (Kober) which is inherent in accurate measuring devices, is obviously a step backwards in colorimeter construction. Another objection to levers is that they are apt to move from a fixed position through jars or accidental touch, no matter how slight.

In 1920 Bausch and Lomb<sup>7</sup> with the aid of Folin brought out a colorimeter of the Duboseq type but which has a number of changes from the French model. Four marked changes are apparent: (1) The base and upright frame are of very heavy castings, "to provide stability and permanent alignment of the optics." (2) The rack and pinion are so changed that the operating heads are always in a fixed position, so that the observer's readings are not influenced by the position of the pinion heads. (3) The colorimeter cups used are of ground glass cylinders and plates, incased in heavy metal. (4) The adjustable verniers of the Kober instrument are adopted. The heavy metal and construction provide an instrument resistant to rough handling, and the fixed position of the milled heads seems to be an improvement over the French model, but the extra massiveness of the parts plays no rôle in its accuracy.

The instrument makers furnish attachments which make it possible to convert it into a nephelometer, after disconnecting plungers and adding these parts. This transformation while not a very convenient one, is of value. The means used to eliminate the meniscus from the nephelometric tubes or vials, and the care taken to eliminate the glare from the light source, which is designed to give parallel rays, are commendable features of this instrument.

During the routine tests of the Kober instruments it has become evident that the physical condition of the operator is an important factor in maintaining a high average of accuracy in adjusting the optical arrangement. It was found that frequent stooping down, in order to read the scale after having matched the colors or light in the eyepiece, induced fatigue very quickly. This stooping is necessary when using any of the Duboseq type instruments. This condition of fatigue was ag-

<sup>6</sup> Advertised by E. Leitz, in *Science*, 1919.

<sup>7</sup> Advertised by Bausch and Lomb, in *J. Ind. and Eng. Chem.*, 1920.

gravated by the difference between the short focal distance in the eyepiece and the longer focus necessary to observe the scale, the result being greater eye fatigue.

Another source of temporary fatigue which even if it is not so pronounced yet is a factor in accurate work, is the holding or supporting of the arms while adjusting or turning milled heads, to operate the plungers or cups. As long as the nephelometer-colorimeter was only used occasionally, these defects of course were not noticeable, but since their use has become daily and in many cases almost continuous, these factors have become important. Many experiments and models were made to eliminate the sources of annoyance and fatigue inherent in the Duboscq type, and although several models achieved the final result, the one described in this paper was adopted as the most satisfactory from all points of view especially since there is no indirect transmission in changing the vertical scale to a horizontal one.

The improvements, which eliminate these defects are: (1) The milled heads, formerly at the top of the instrument, are placed at the bottom, which allows the hands to rest on the table or other support and the adjustments to be made with the fingers (shown in Fig. 1). (2) An auxiliary scale is provided at the top of the instrument consisting of: two scales engraved upon the side away from the operator, fastened to the movable stages, so that when the stage is being moved up or down, the scales move with it; a stationary vernier, protruding beyond the top plate, also engraved upon the side away from the operator, fastened to the top of the instrument. A mirror facing the operator at an angle of  $45^\circ$  is placed in front of the protruding scale and vernier, so that an image of the two is reflected vertically. A magnifying glass of the same focal distance as the telescope, serving as a second eyepiece, has been placed close beside the regular eyepiece, directly above the mirror, showing the image of the scale enlarged in good light.

Fig. 1 shows the entire instrument without the lamp house.<sup>8</sup>

<sup>8</sup> This instrument is manufactured by Klett Manufacturing Company, Inc., 202 East 46th Street, New York.

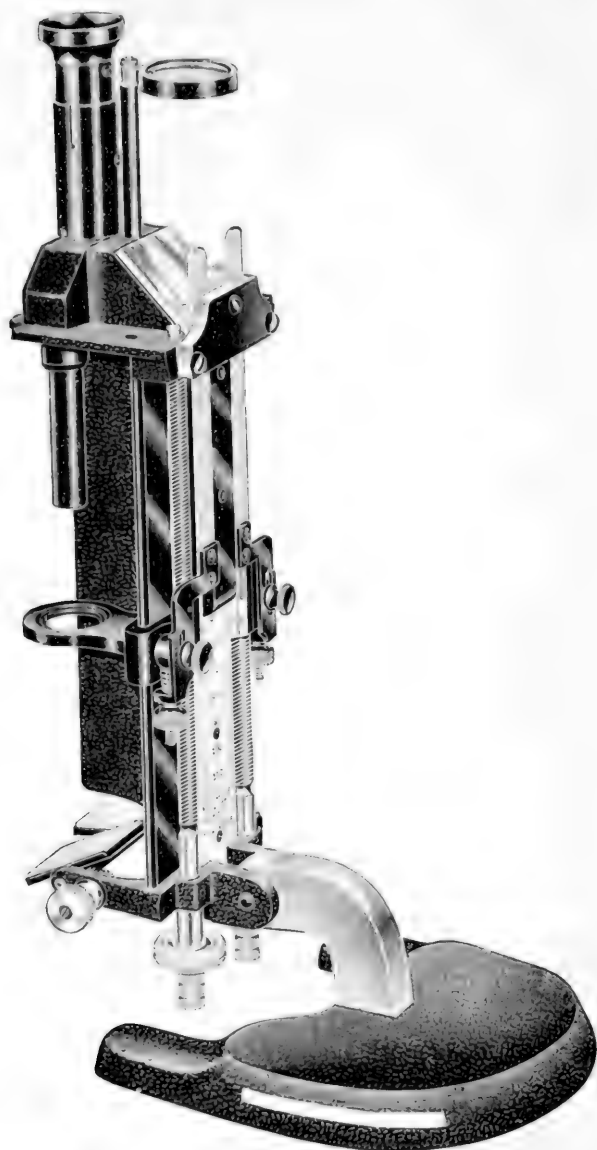


FIG. 1. View of the instrument without the lamp house, showing (a) the two eyepieces, the scale, and the mirror at the top of the instrument; (b) the micrometer adjustment of the zero point; (c) the milled heads operating the cups at the bottom of the instrument.

In Fig. 2 are shown the fields that are observed through the two eyepieces. The ease of reading the scale is apparent.

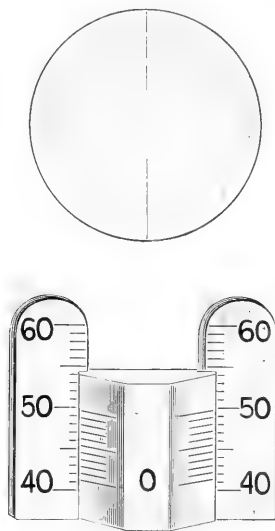


FIG. 2. The two fields as seen through the two eyepieces. The upper field shows the two semicircular fields; the lower field shows the stationary vernier and the two adjustable scales.

In Fig. 3 is shown the construction of the auxiliary scale. This auxiliary scale is engraved to 60 mm. but with the vernier is readable to only 50 mm., which is ample for most work. If heights above 50 mm. are to be measured the original vernier can be used. The setting of the zero point is easily and accurately accomplished with a micrometer arrangement, as may be seen at A by a milled head working against a spring. This convenient method of zero point adjustment, together with the very simple method of using the instrument, the method of Lamb, Carleton, and Meldrum,<sup>9</sup> where the height of the standard solution (S) is kept constant, makes the operation of the instrument and the calculation of results extremely simple and easy without, however, sacrificing accuracy or deviating from the fundamental basis of either colorimetry or nephelometry.

<sup>9</sup> Lamb, A. B., Carleton, P. W., and Meldrum, W. B., *J. Am. Chem. Soc.*, 1920, xlii, 252.

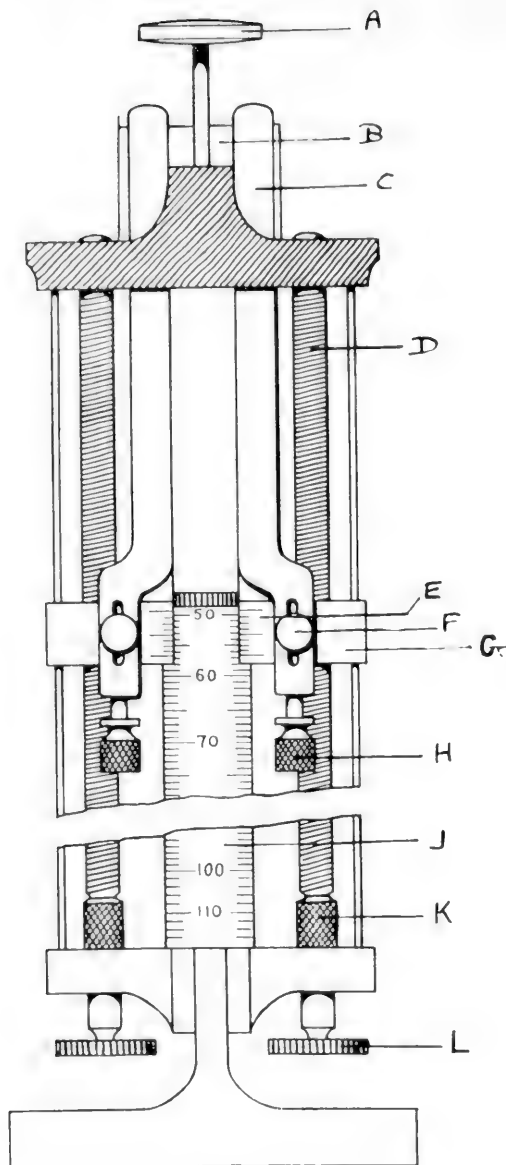


FIG. 3. Diagrammatic sketch of the rear of the instrument. A, magnifying lens for the horizontal scale; B, mirror at an angle of  $45^\circ$ ; C, movable scale; D, screw-threaded rod; E, vernier for 50 to 100 mm. scale; F, lock screw for zero adjustment; G, movable cup carrier; H, micrometer for zero adjustment; J, scale from 50 to 100 mm.; K, knurled thumb screw for rapid movement; L, knurled thumb screw for fine adjustment.



In Fig. 4 is shown the instrument attached to a lamp house.

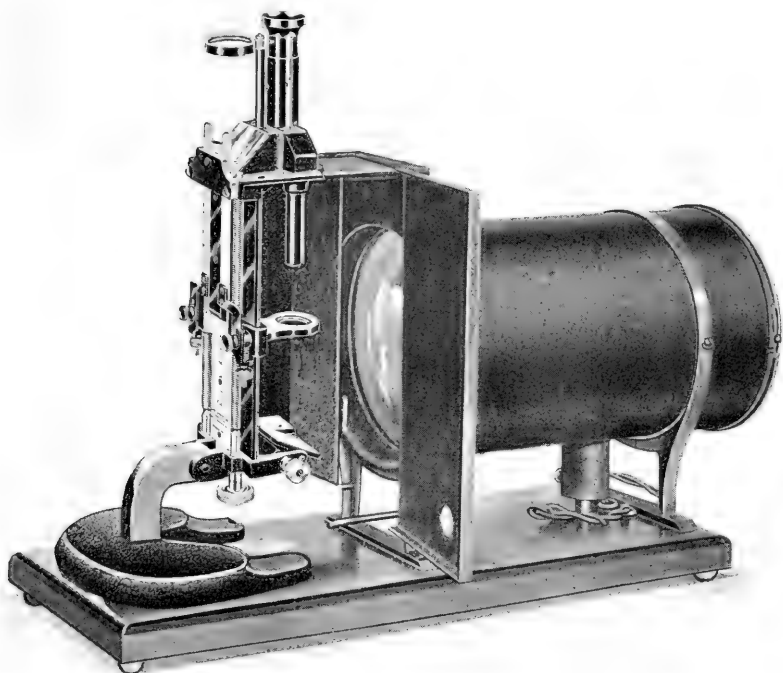
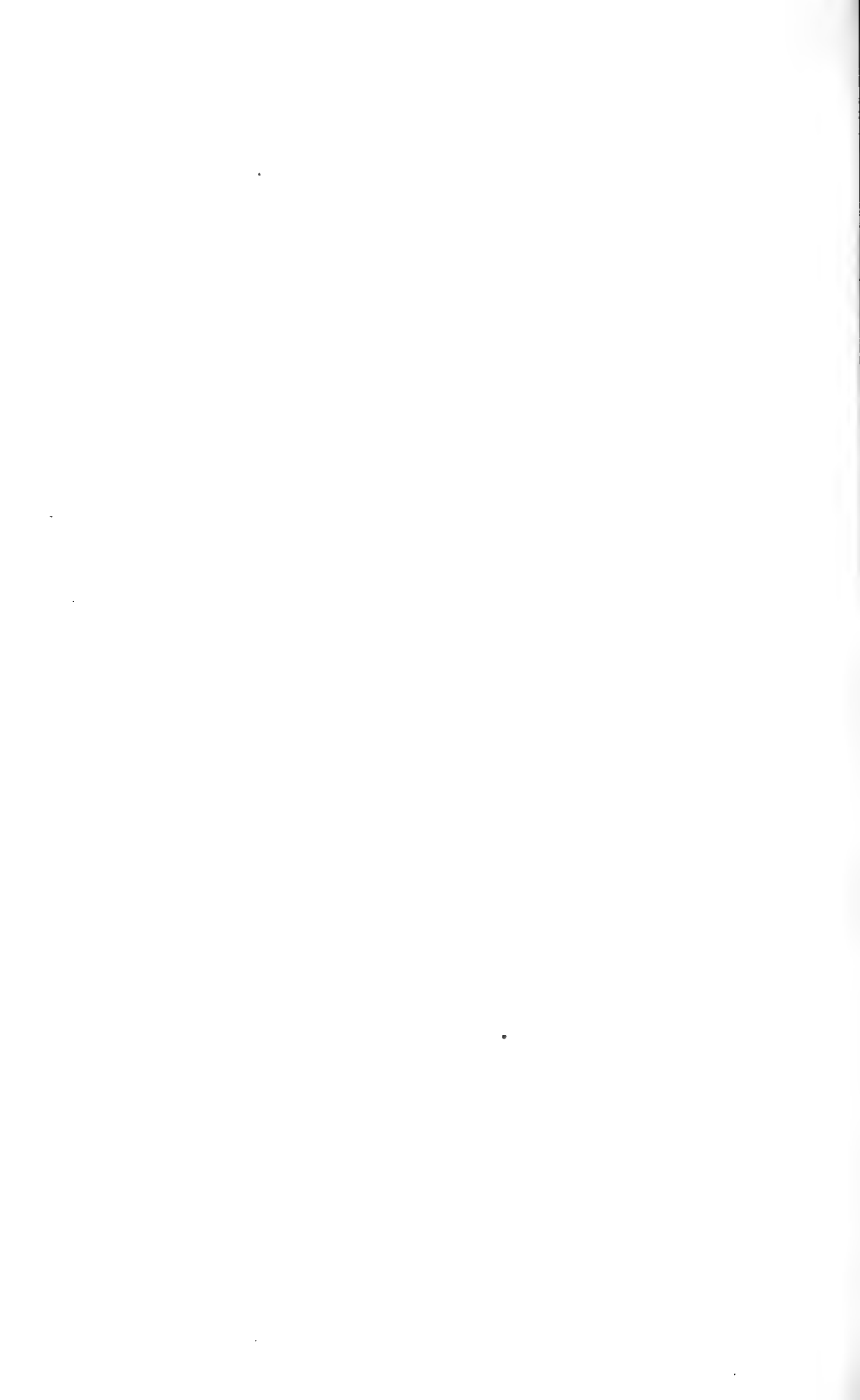


FIG. 4. The instrument and lamp house showing the split reflectors as well as the front of the instrument illuminated by the light from the lamp house.

#### SUMMARY.

The advantages of the new improvements are: (1) the elimination of the fatigue and annoyance, due to stooping to read the scale of Duboseq instruments; (2) an enlarged and well illuminated scale read through an eyepiece of the same focal length as the telescope; (3) a more convenient position for the milled heads operating the stages, allowing for resting of the operator's arms; and (4) a micrometer arrangement for setting the zero point which can be locked in any position.



# ON THE SUBSTITUTION OF TURBIDIMETRY FOR NEPHELOMETRY IN CERTAIN BIOCHEMICAL METHODS OF ANALYSIS.

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(Received for publication, April 25, 1921.)

Within the past decade nephelometric methods of analysis have become increasingly popular in biochemical work, so that at present a nephelometer has come to be counted an indispensable piece of apparatus in every well equipped laboratory. Nephelometry has however the serious disadvantage that, as was first pointed out by Richards and Wells,<sup>1</sup> the amount of light reflected is not strictly proportional to the weight of the precipitate under observation, but seems to be influenced by a variety of factors. To overcome this defect Richards and Wells adjusted the volume of their solutions so that standard and unknown contained about the same concentration. Kober<sup>2</sup> has suggested a mathematical formula by means of which he has obtained excellent results, and Bloor<sup>3</sup> recommends that a standard be selected which is of such strength that it varies not more than 25 per cent from the unknown.

During the past 5 years the author has had occasion, in connection with studies on blood and milk, to make extensive use of nephelometric methods, and as a result of this experience has come to appreciate more clearly the relatively large error which may be introduced in an analysis unless the strength of the standard and of the unknown are adjusted to within at least 10 per cent, a requirement which means that for a single determination it may be necessary to provide from three to six stan-

<sup>1</sup> Richards, T. W., and Wells, R. C., *Am. Chem. J.*, 1904, xxxi, 235.

<sup>2</sup> Kober, P. A., *J. Biol. Chem.*, 1912-13, xiii, 485.

<sup>3</sup> Bloor, W. R., *J. Biol. Chem.*, 1918, xxxvi, 33.

dards of varying degrees of concentration. The irksomeness of preparing large numbers of standards has led to an investigation concerning the possibility of utilizing the turbidimeter in place of the nephelometer in several biochemical methods of analysis.

Turbidimetric methods of analysis are extensively used in technical work, as for example in connection with the determination of suspended matter in water, of sulfur in coal, etc., but so far but little use has been made of this principle in the solution of biochemical problems. Some years ago Folin and Denis<sup>4</sup> made use of this technique for the determination of albumin in urine, and recently Denis and Ayer<sup>5</sup> have employed a similar method in the analysis of cerebrospinal fluid. In connection with the above work, and as the result of a series of readings made on standard solutions of widely varying concentrations we felt justified in believing that turbidity determinations made on the precipitate obtained by the action of sulfosalicylic acid on protein give comparable results even when the concentration of the unknown and of the standard vary as much as 50 per cent. If it could be proved that this finding also applies to the measurement of suspensions other than protein, the usefulness of the turbidimeter in biochemical work becomes immediately apparent.

Although several types of turbidimeters have been suggested, I have continued the use of the Duboseq colorimeter for the measurement of my suspensions. To obtain the best results all readings should be made in a dark or semidark room, and as a preliminary to any series of readings the position of the colorimeter should be so adjusted with relation to the source of light, that exactly the same illumination is obtained on both sides when both cups are filled with the standard suspension and both scales are set at the same point. It has been my experience that the most accurate results are secured when the mirror is adjusted to give the maximum illumination.

In this paper I wish to present the results of a study which has for its basis the attempt to substitute turbidimetry for nephelometry in three analytical procedures; *viz.*, the determination of

<sup>4</sup> Folin, O., and Denis, W., *J. Biol. Chem.*, 1914, xviii, 273.

<sup>5</sup> Denis, W., and Ayer, J. B., *Arch. Int. Med.*, 1920, xxvi, 436.

calcium in blood by Lyman's method<sup>6</sup> (in which the blood calcium in the form of colloidal calcium stearate is measured by comparison with a standard calcium stearate suspension); the determination of fat in blood and milk by the method of Bloor<sup>7</sup> (which involves the use of colloidal suspensions of fatty acids); and of phosphates by the strychnine molybdate precipitate of Pouget and Chouchak<sup>8</sup> as modified by Kober and Egerer<sup>9</sup> and by Bloor.<sup>3</sup>

The results obtained with calcium stearate suspensions indicate that it is easily possible to obtain quantitative results with suspensions varying in concentration by as much as 50 per cent, provided these suspensions are of such concentration that they contain between 0.75 and 0.35 mg. of calcium in a final volume of 100 cc.; with greater concentration precipitation frequently occurs; while if the amount of calcium is less than 0.35 mg. per 100 cc. turbidimetric readings can no longer be made with precision.

TABLE I.

*Comparison of Results Obtained by the Use of the Nephelometer and the Colorimeter in the Determination of Calcium in Milk.*

Observation No.	Ca per 100 cc. of milk.	
	By nephelometry.	By turbidimetry.
	<i>mg.</i>	<i>mg.</i>
210	18.6	18.6
237	22.1	22.9
239	20.0	20.0
235	19.2	19.4
240	33.3	33.1
269	21.6	21.0
232	17.2	17.4

In Table 1 are presented the results obtained in a series of determinations of calcium in milk by Lyman's method in which parallel readings were made by the turbidimeter and by the nephelometer.

<sup>6</sup> Lyman, H., *J. Biol. Chem.*, 1917, xxix, 169.

<sup>7</sup> Bloor, W. R., *J. Biol. Chem.*, 1914, xvii, 377.

<sup>8</sup> Pouget, I., and Chouchak, D., *Bull. Soc. chim.*, 1909, v, series 4, 104.

<sup>9</sup> Kober, P. A., and Egerer, G., *J. Am. Chem. Soc.*, 1915, xxxvii, 2373.

With fatty acid suspensions quantitative readings can be made when the standard and unknown vary by 60 per cent provided the concentrations of fatty acid lie within the range of 8 to 2 mg. per 100 cc.

In Table II comparison is made of analyses of milk fat made by the Babcock method, and by Bloor's method, readings having been made by means of the turbidimeter instead of the nephelometer.

TABLE II.

*Comparison of Results Obtained by Determinations of Fat in Milk by the Babcock Procedure and by the Use of the Colorimeter in Bloor's Micro Fat Method.*

Observation No.	Fat.	
	By Babcock method.	By turbidity.
	<i>per cent</i>	<i>per cent</i>
1	3.2	3.2
2	3.1	3.0
3	3.5	3.6
4	4.3	4.4
5	4.7	4.4
6	5.0	5.1

TABLE III.

*Comparison of Results Obtained by the Use of the Nephelometer and the Colorimeter in the Determination of Inorganic Phosphates in Blood Plasma.*

Observation No.	P per 100 cc. of plasma.	
	By nephelometer.	By colorimeter.
	<i>mg.</i>	<i>mg.</i>
143	2.3	2.2
144	2.6	2.7
163	3.0	3.0
162	3.8	2.6
160	5.0	5.9
247	6.2	6.0

Results, essentially similar to those reported above, were obtained with strychnine phosphomolybdate suspensions. The optimum concentrations for turbidimetric work with this material were found to be 0.12 to 1.2 mg.  $\text{H}_3\text{PO}_4$  per 100 cc.

In Table III are collected the results of a series of determinations of the inorganic phosphates of blood plasma in which parallel readings were made by the nephelometer and by the turbidimeter.

#### SUMMARY.

The suggestion is made that determinations of turbidity, made by means of a colorimeter, may with advantage be substituted for nephelometric readings in several analytical processes. The advantages of this procedure are twofold: first, as turbidimetric readings give quantitative results with large variations in concentration between standard and unknown it is possible to omit the preparation of the large number of standards which have been found necessary in nephelometric work; second, it presents an additional use for the now universally owned colorimeter and in the case of many small laboratories would probably make the possession of a nephelometer unnecessary.





## CREATINURIA.

### II. ARGININE AND CYSTINE AS PRECURSORS OF CREATINE.\*

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(Received for publication, April 20, 1921.)

In a previous publication (1) we have analyzed the various factors which may be operative in the production and prevention of creatinuria. This analysis was suggested by the possibility that the anomalies observed in the appearance of creatinuria could very well owe their causation to the variation in the utilization of amino-acid precursors of creatine with different physiological states or functions. Processes of growth, fetus building, and lactation would have their influence on creatinuria in modifying the intake and utilization of amino-acids and therefore varying the residue left to be catabolized into creatine. Creatine excretion could thus be indicated, not only as a result of excessive ingestion of its precursors, but also as a result of deficient ingestion of the complements of the precursors necessary to make protein building possible. Excessive catabolism such as obtains during fasting, fever, acidosis, and phosphorus poisoning would then similarly owe its creatinuria to the excessive liberation of creatine precursors although in part some of the creatine excreted would originate from the creatine normally found as a constituent of muscle.

Our previous investigations designed to correlate some of these factors have shown that creatinuria can always be induced in the pig by feeding sufficient protein. This was found to be the case irrespective of the sex of the animal, or the acidity or alkalinity of the diet. As a result we have continued our study of the problem in an attempt to define further its origin.

\* Published with the permission of the Director of the Wisconsin Agricultural Experiment Station.

In the search for possible precursors of creatine, arginine has attracted the most attention because, like creatine, it is a derivative of guanidine and because it is contained in all the known proteins. In fact it is the only cleavage product of protein so far isolated which contains the guanidine nucleus.

While it is now generally accepted that creatine is derived from proteins, such a difference of opinion exists in the literature as to the possibility of arginine being the precursor of creatine, that no definite conclusion as to the exact state of affairs can be drawn. Direct experimentation with the feeding of arginine has been rather limited but considerable work has been done by transfusion and injection. Inouye (2) found an increase in creatine upon adding arginine to liver extract, and also when arginine was perfused through the surviving liver. Myers and Fine (3) in feeding rats with edestin, which contains 14 per cent of arginine, and casein, which contains 4 per cent of arginine, found a slight increase in the muscle creatine with the former, but they draw no conclusions. Thompson (4) reported an increase in urinary creatine following injection of arginine carbonate, but failed to obtain an increase by oral administration. Jaffé (5) found no increase in urinary creatine following the feeding of arginine to the rabbit.

Recently Harding and Young (6) have suggested that cystine might also be metabolized into creatine "through the intermediate stages of taurine and amino ethyl alcohol, followed by methylation, combination with urea, and oxidation." Using dogs for the experiments they obtained increases with cystine but not with arginine. It appeared significant to us that in their report they state that the endogenous creatine was affected as well as the exogenous. This suggested that possibly what was interpreted by them as a direct effect of cystine was in reality an indirect effect—the cystine stimulating catabolism by means of the sulfuric acid formed by its oxidation. Of their failure with arginine we will have more to say later.

In this paper there will be presented data showing how arginine and cystine may function in inducing or augmenting creatinuria.

## EXPERIMENTAL.

In the following experiments as in the past, the pig was chosen as the experimental animal as it stands confinement well and can be fed a nitrogen-free diet over a long period of time. Such a diet offers especial advantages in that it simplifies the conditions of experimentation and allows more accurate interpretation of the data. Methods of analysis and the experimental procedure used were the same as before (1). Total nitrogen and creatinine determinations were made to serve as an index of general excretory activity.

The arginine used was prepared according to Kossel's method from the protein arachin. As a check on its purity it was analyzed according to Van Slyke. To remove traces of barium which always tend to be carried along in the preparation, sodium sulfate was added in excess. In later experiments, Tables IV and V, however, the barium was removed quantitatively with sulfuric acid as it was feared that the sodium carbonate formed in the other procedure might affect the results.

The arginine was usually given by stomach tube. In the experiments of Table IX it was given in gelatin capsules. Usually some difficulty was experienced in getting the pig to retain it as it appeared to be nauseating. Some success was had in neutralizing or acidifying it with acetic or lactic acid but even with this precaution the largest amount that could be given without always causing vomiting or diarrhea was the equivalent of 100 gm. of casein.

The cystine given was prepared from human hair by acid hydrolysis. Analyses showed it to contain 26.63 per cent sulfur, the theoretical being 26.69 per cent. It was administered in water suspension by stomach tube.

Not all the pigs were found suitable for the demonstration of the effect of these amino-acids. In some the production of creatine was so low that excessive casein, 300 to 400 gm. had to be fed before the threshold for creatine excretion was reached. As the arginine equivalent of such amounts of casein could not be successfully given these animals were discarded. With all pigs, preliminary to the amino-acid administrations, casein was given for purposes of comparison as to the amounts of creatine produced.

*Experiments with Arginine.*

TABLE I.

Pig, male, weight 28 kilos. Energy intake, 75 calories per kilo.

Day of starch feeding.	Volume of urine.	Urinary nitrogen.	Pre-formed creatinine.	Creatine as creatinine.	Diet.
	cc.	gm.	gm.	gm.	
11	1,550	4.40*	0.857	0.068	Starch 500 gm.
12	1,400	2.57	0.945	0.044	" 500 "
13	1,225	3.62	0.772	0.168	" 500 " arginine 4.08 gm.
14	1,575	2.52	0.849	0.000	" 500 "
15	1,300	2.10	0.819	0.084	" 500 "
16	1,450	2.32	0.804	0.073	" 500 "

\* The high urinary nitrogen represents in part residual nitrogen from an attempt to feed 200 gm. of casein 2 days previously.

In Table I is shown the effect of arginine on creatine production. Previous to the collection of the data here presented, observations had been taken on the effect of casein administration. When 100 gm. of casein were given, after 4 days maintenance on a starch diet, the creatine increased from an average value of 60 to 103 mg. An attempt to further increase the excretion by giving 200 gm. of casein resulted in partial anorexia. The nitrogen excretion on the first day bears evidence, however, as shown in the table, that the casein had been partially consumed.

TABLE II.

Pig, female, weight 24 kilos. Energy intake, 75 calories per kilo.

Day of starch feeding.	Volume of urine.	Urinary nitrogen.	Pre-formed creatinine.	Creatine as creatinine.	Diet.
	cc.	gm.	gm.	gm.	
8	1,450	1.94	0.460	0.053	Starch 400 gm.
9	2,200	2.20	0.344	0.073	" 400 "
10	1,200	5.54	0.342	0.163	" 300 " casein 100 gm.
11	1,200	2.80	0.330	0.065	" 400 "
12	1,250	1.85	0.485	0.075	" 400 "
13	1,450	2.92	0.468	0.087	" 400 " arginine 4.08 gm.
14	1,550	1.92	0.451	0.121	" 400 "
15	1,650	1.88	0.478	0.051	" 400 "
16	1,500	1.68	0.505	0.085	" 400 "

In Table II is brought out the effect of casein and arginine in creatine production. In both cases the creatine excretion was increased, although after arginine feeding, the increase was not observed until the second day. This retardation in excretion has also been noted after casein feeding, even though the major portion of the nitrogen was excreted on the first day. We believe this is indicative of the fact that we are dealing with a special form of amino-acid metabolism which is independent, at least to a considerable extent, of the major processes of protein metabolism.

TABLE III.

Pig, male, weight 25 kilos. Energy intake, 65 calories per kilo.

Day of starch feeding.	Volume of urine.	Urinary nitrogen.	Pre-formed creatinine.	Creatine as creatinine.	Diet.
	cc.	gm.	gm.	gm.	
20	1,850	1.59	0.410	0.121	Starch 400 gm.
21	1,800	1.33	0.427	0.090	" 400 "
22	1,850	3.95	0.415	0.238	" 300 " casein 100 gm.
23	1,800	2.48	0.382	0.122	" 400 "
24	2,000	1.76	0.384	0.112	" 400 "
25	1,750	3.11	0.398	0.162	" 400 " arginine 4.08 gm.
26	1,800	1.40	0.382	0.122	" 400 "
27	2,000	1.52	0.368	0.100	" 400 "
28	1,850	3.14	0.415	0.278	" 300 " casein 100 gm.
29	2,350	2.39	0.305	0.103	" 400 "
30	1,850	1.36	0.360	0.125	" 400 "

Table III again shows a distinct, though small, rise in creatine excretion after arginine administration. It is not comparable to the rise obtained on casein of equal arginine content. In fact, this is what is generally observed. The arginine given in this experiment was neutralized with lactic acid, instead of acetic acid, after sodium sulfate had been added to remove the barium.

Tables IV and V also bear testimony to the fact that arginine as well as casein can increase the creatinuria. These tables are presented as examples of what has been obtained time and again, none of them showing any great uniformity in creatine production. Sometimes a rise of 30 mg. was obtained, then again one

of 115 mg., and occasionally none at all. This latter observation is not to be taken as one which invalidates our contention that arginine is a precursor of creatine as in such instances a drop

TABLE IV.

Pig, male, weight 28 kilos. Energy intake, 75 calories per kilo.

Day of starch feeding.	Volume of urine.	Urinary nitrogen.	Pre-formed creatinine.	Creatine as creatinine.	Diet.
	cc.	gm.	gm.	gm.	
5	1,600	1.76	0.507	0.052	Starch 500 gm.
6	1,800	1.43	0.534	0.036	" 500 "
7	1,750	1.57	0.585	0.055	" 500 "
8	2,000	2.25	0.595	0.045	" 500 "
9	1,550	3.13	0.541	0.192	" 400 " casein 105 gm.*
10	2,000	2.40	0.695	0.115	" 500 "
11	2,100	1.34	0.598	0.086	" 500 "
12	1,850	2.59	0.654	0.154	" 500 " arginine 4.08 gm.
13	1,850	1.83	0.675	0.058	" 500 "

\* 105 gm. of this casein was required to furnish the same amount of nitrogen as 100 gm. before.

TABLE V.

Pig, male, weight 29 kilos. Energy intake, 70 calories per kilo.

Day of starch feeding.	Volume of urine.	Urinary nitrogen.	Pre-formed creatinine.	Creatine as creatinine.	Diet.
	cc.	gm.	gm.	gm.	
9	2,100	1.47	0.430	0.042	Starch 500 gm.
10	2,600	1.72	0.505	0.070	" 500 "
11	2,200	2.44	0.533	0.182	" 400 " casein 105 gm.
12	1,650	2.31	0.539	0.000	" 500 "
13	1,700	2.72	0.489	0.131	" 500 " arginine 4.08 gm.
14	2,300	2.34	0.517	0.195	" 500 "
15	2,700	1.72	0.561	0.060	" 500 "
16	2,200	1.64	0.616	0.066	" 500 "
17	2,500	2.20	0.415	0.205	" 500 " arginine 4.08 gm.
18	2,400	1.92	0.479	0.143	" 500 "
19	1,800	1.76	0.468	0.072	" 500 "

in the excretion independent of dietary influences may have occurred and the arginine administered merely counterbalanced this decrement. Such changes, irrespective of dietary modifi-

cations, are very common, in fact, in some pigs the creatinuria may disappear entirely only to reappear upon the administration of very small amounts of protein. Apparently the production of creatine in these cases has merely fallen below the threshold of its excretion.

As a general occurrence, more creatine was excreted after casein feeding than after the administration of its arginine equivalent—the former exceeding the latter by about 25 mg. or 25 per cent. This is hardly surprising when we take into consideration the fact, that acids, such as phosphoric acid which is liberated in the metabolism of casein, stimulate the production of creatinuria, but two other possibilities also suggest themselves. In the first place, the casein molecule may carry still other precursors of creatine to augment its excretion, and in the second place, it is possible that free arginine may be metabolized to urea faster—and thus circumvent its formation into creatine—than the arginine as absorbed with the products of digestion. These are mere hypotheses of which only the first one can derive any support from facts now available.

#### *Experiments with Cystine.*

In the studies of the production or augmentation of creatinuria by cystine feeding, analyses of the urines for total sulfur and sulfates as well as for total nitrogen, creatine, and creatinine were made. Total sulfur was determined by Benedict's method and total sulfates by Folin's method. 4.08 gm. of cystine—the same amount as arginine previously—were given to the pig in each trial.

In Tables VI and VII it is seen that cystine feeding increased the creatinuria to about the same degree as, or possibly slightly more, than the same amount of arginine. In Table VI after the first cystine administration the rise in creatine was delayed 1 day. This is similar to what we have seen with arginine and possibly indicates that the creatine is subject to special laws of excretion in comparison with the total nitrogen, sulfur, and other compounds. The cystine sulfur makes its appearance in the urine vary largely in the oxidized form, showing a tendency to a disturbance of the acid-base balance. Acidity determinations were not made.

TABLE VI.

This record is a continuation of that shown in Table IV, the same pig being used throughout. 500 gm. of starch were fed daily.

Day of starch feeding.	Volume of urine.	Urinary nitrogen.	Pre-formed creatinine.	Creatine as creatinine.	Total sulfur.	Total sulfate sulfur.	Neutral sulfur.	Dietary addition.
	cc.	gm.	gm.	gm.	gm.	gm.	gm.	
14	2,200	1.27	0.533	0.094	0.475	0.255	0.220	
15	2,200	1.45	0.550	0.027	0.446	0.279	0.166	
16	1,800	1.40	0.575	0.052	0.399	0.234	0.162	
17	2,450	2.53	0.587	0.038	1.029	0.718	0.310	Cystine 4.08 gm.
18	2,150	1.41	0.596	0.150	0.627	0.462	0.165	
19	1,950	1.48	0.472	0.039	0.764	0.580	0.184	
20	1,950	2.34	0.589	0.087	0.593	0.378	0.215	
21	2,300	2.85	0.644	0.132	1.110	0.668	0.442	Cystine 4.08 gm.
22	2,150	1.72	0.612	0.039	0.602	0.385	0.217	
23	1,950	1.82	0.560	0.062				

TABLE VII.

This record is a continuation of that shown in Table V, 3 days' collection being omitted. 500 gm. of starch were fed daily.

Day of starch feeding.	Volume of urine.	Urinary nitrogen.	Pre-formed creatinine.	Creatine as creatinine.	Total sulfur.	Total sulfate sulfur.	Neutral sulfur.	Dietary addition.
	cc.	gm.	gm.	gm.	gm.	gm.	gm.	
23	2,100	1.72	0.582	0.053	0.359	0.310	0.048	
24	2,100	1.93	0.619	0.063	0.462	0.277	0.184	
25	2,150	2.66	0.541	0.155	0.673	0.364	0.309	Cystine 4.08 gm.
26	2,200	2.24	0.632	0.083	0.712	0.495	0.217	
27	2,000	2.04	0.650	0.100	0.535	0.351	0.184	
28	2,200	1.76	0.577	0.066	0.485	0.323	0.162	
29	2,150	2.15	0.617	0.129	0.939	0.558	0.384	Cystine 4.08 gm.
30	2,400	2.18	0.538	0.089	0.683			
31	2,300	1.93	0.542	0.058	0.618	0.367	0.251	



Table VIII shows how creatinuria is influenced by acidosis. Normally on a starch diet the urine of a pig is always acid. Under such conditions, with this particular animal creatine was always excreted. When the urine was changed to an alkaline reaction by sodium acetate administration the creatine promptly disap-

TABLE VIII.

Pig, male, weight 40 kilos. Energy intake in the form of starch, 75 calories per kilo except when casein was given; then an equivalent isodynamic reduction in starch intake was made.

Day of starch feeding.	Volume of urine.	Urinary nitrogen.	Preformed creatinine.	Creatine as creatinine.	Total sulfur.	Total sulfate sulfur.	Neutral sulfur.	Dietary addition.
	cc.	gm.	gm.	gm.	gm.	gm.	gm.	
14	2,300	2.25	1.177	0.101				
15	1,050	1.98	1.344	0.115	0.424	0.329	0.095	Sodium acetate 50 gm.
16	1,000	2.32	1.470	0.000	0.508			" " 50 "
17	2,000	2.72	1.200	0.000	0.560	0.320	0.240	" " 50 "
18	2,250	2.92	1.327	0.000	1.417	0.732	0.685	" " 50 " cystine 4.08 gm.
19	2,150	2.27	1.128	0.000	0.691	0.645	0.046	Sodium acetate 20 gm.
20	1,750	5.32	1.235	0.222	0.937	0.576	0.361	" " 50 " casein 165 gm.
21	1,800	2.11	1.151	0.000	0.335	0.343	0.191	Sodium acetate 25 gm.
22	2,800	1.48	1.109	0.000	0.806	0.761	0.045	" " 25 "
23	2,800	3.81	1.299	0.000	0.576	0.375	0.201	" " 50 " cystine 4.08 gm.
24	2,700	2.43	1.296	0.000	0.475	0.278	0.197	Sodium acetate 25 gm.
25	2,100	2.56	1.207	0.000	1.032	0.777	0.265	" " 50 " cystine 4.08 gm.
26	3,100	2.70	1.010	0.000	0.750	0.540	0.210	Sodium acetate 25 gm.
27	2,400	2.44	1.190	0.000	1.190	1.040	0.150	" " 50 " cystine 4.08 gm.
28	3,000	2.10	1.080	0.000	0.422	0.308	0.014	Sodium acetate 25 gm.
29	1,800	5.18	1.212	0.168	0.765	0.582	0.183	" " 50 " casein 165 gm.
30	3,000	6.06	1.033	0.080	0.525	0.315	0.210	Sodium acetate 25 gm.

peared. Under these conditions cystine feeding was without effect in each of the four individual trials attempted; casein, on the other hand, was effective as usual. This seems to prove beyond a question that cystine creatinuria owes its origin to other factors than those operative when casein is fed.

TABLE IX.

A continuation of Table VIII with the same dietary régime but with other additions, comparing the effect of arginine with cystine administrations on creatinuria production.

Day of starch feeding.	Volume of urine.	Urinary nitrogen.	Pre-formed creatinine.	Creatine as creatinine.	Dietary additions.
	cc.	gm.	gm.	gm.	
31	2,150	3.27	1.131	0.000	Sodium acetate 25 gm.
32	2,150	3.39	1.139	0.140	" " 25 " arginine 4.08 gm.
33	2,950	3.00	1.130	0.000	Sodium acetate 25 gm.
34	2,500	3.50	1.060	0.090	" " 25 " arginine 4.08 gm.
35	3,000	3.12	1.110	0.040	Sodium acetate 25 gm.
36	3,100	3.03	1.187	0.000	" " 25 "
37	3,600	2.52	1.034	0.000	" " 50 " cystine 4.08 gm.
38	3,850	3.08	1.155	0.000	
39	3,000	2.64	1.010	0.000*	
40	3,050	2.44	1.097	0.000†	
41	2,800	2.63	1.065	0.071‡	
42	3,400	2.65	1.144	0.067	
43	3,300	2.64	1.056	0.090	
44	3,500	3.57	1.038	0.160	Cystine 4.08 gm.
45	2,200	2.11	0.990	0.000	

\* The urine, since the first administration of sodium acetate (Table VIII) had always been kept alkaline to phenolphthalein. On this day with the cessation of sodium acetate feeding it was acid to phenolphthalein but alkaline to litmus.

† Urine was neutral to litmus.

‡ Urine was acid to litmus on this day and subsequently.

The relations brought out in Table VIII are emphasized in their significance by the data presented in Table IX where creatinuria, when induced by arginine feeding is also shown not to be affected by sodium acetate administration. Under such conditions cystine was again shown to be unable to produce creatinuria as long as the urine remained alkaline. After the urine had become acid to litmus again creatine reappeared and increased in amount with cystine administration. Failure of its continued appearance is left unexplained as the experiment was terminated, but probably represents one of the variations in creatine excretion called attention to before.

## SUMMARY.

Arginine administered orally in sufficient amounts augments creatine excretion in the pig.

Creatinuria induced by casein feeding appears to have its origin in large part in the formation of creatine from arginine, but the acidity of the phosphoric acid split off no doubt also contributes to the creatinuria as a result of the stimulation of endogenous metabolism.

Cystine feeding causes creatinuria only when the sulfuric acid formed by the oxidation of its sulfur is left unneutralized; when neutralized the creatinuria promptly disappears.

Neutralization of acidity does not prevent the creatinuria called forth by casein or arginine feeding.

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## CREATINURIA.

### III. THE EFFECT OF THYROID FEEDING UPON CREATINURIA.\*

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(Received for publication, April 20, 1921.)

The study of any reaction in the animal body, where only the excretory end-products are available to indicate the course of the whole, is bound to reveal many apparent inconsistencies. Thus in attributing the origin of creatine to certain processes of protein metabolism, investigators are confronted with the undeniable fact that the most drastic attempts to induce creatinuria in men by protein feeding have met with failure. With women no such difficulty has been encountered. In our experiments (1) carried out with the pig no difficulty in the production of creatinuria by protein feeding has manifested itself, irrespective of the sex of the animal. We have, however, observed certain variations among individuals. For instance, some pigs do not excrete creatine in their urine when fed a starch diet or when on a normal ration, but when fasted for a number of weeks such individuals may or may not show the presence of creatine. We have also observed a great variation in the amount of casein necessary to produce creatinuria in the animals. Some require 100 gm., and others require as much as 300 gm. or more. Differences in behavior might be taken as an index of the nearness of approach of endogenous creatine production—though the exogenous would present similar relations—to the threshold level of its excretion. Yet even this does not tell the whole story as animals already showing creatinuria on a nitrogen-free diet often require more than 100 gm. of casein before any demonstrable increase in creatinuria results. Creatine precursors of exogenous origin evidently are submitted to a different array of metabolic processes from those originating endogenously. Otherwise, very

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small amounts of casein should suffice to increase a creatinuria already in existence. As a matter of fact Thompson (2) actually found that while he was unable to increase creatinuria in certain dogs by feeding arginine, upon injecting it subcutaneously 9 per cent was recoverable as creatine. Normally we believe that the great bulk of creatine formed is metabolized into creatinine and thus kept from accumulating in the blood stream to the level at which its excretion would become possible, but as this reaction is relatively constant among individuals and is not modified except by the administration of tremendous amounts of creatine it undoubtedly is a factor of little importance in the problem under consideration. The main possibility demanding our attention is the fact that creatine is formed or not formed in direct proportion to the balance that obtains between the arginase system, destructive as far as creatine formation is concerned, and the oxidative system whereby the guanidine grouping is left intact. A change in the velocity of either would immediately affect the end-result.

Under ordinary conditions the arginase reaction appears to be very prominent as the administration of arginine results in creatine formation to the extent of only 3 or 4 per cent of the theoretical possibility as shown in our experiments (3). Of the nature of the other mechanism practically nothing is known.

Denis (4) in 1917 reported the production of creatinuria, in a man afflicted with Graves disease, by protein feeding. As it is well known that in this malady the rate of metabolism is enormously increased it suggested itself to us that possibly the thyroid mechanism might be responsible for the differences in the result of protein feeding to normal men as compared with women. As the active principle, thyroxin, of the thyroid gland functions in oxidative reactions it appears possible that it may take part in influencing creatinuria in oxidizing arginine, thus removing it from the sphere of activity of arginase and increasing the amount of creatine formed. On the basis of these hypotheses an attempt was made to study the effect of feeding thyroid preparations on creatinuria of both exogenous and endogenous origin.

While a vast amount of information, relative to oxygen consumption and carbon dioxide production under thyroid influences,

has been collected, surprisingly little work appears to have been done on the effect of thyroid secretion or preparations on nitrogen metabolism beyond establishing an increased nitrogen excretion. This has been observed by Magnus-Levy (5) and Andersson and Bergman (6) on feeding thyroid to normal men. Schöndorff (7), Gluzinski and Lemberger (8), and Voit (9) observed it with dogs and Farrant (10) with cats and rabbits. Underhill and Saiki (11) found but a slight increase in urinary nitrogen as a result of thyroid feeding with no change in urinary nitrogen distribution. Cramer and Krause (12), on the other hand, obtained an increased creatinuria with both men and dogs as the result of artificially induced hyperthyroidism.

TABLE I.

Pig, male, weight 31.5 kilos. Energy intake as starch, 65 calories per kilo.

Day of starch feeding.	Volume of urine.	Urinary nitrogen.	Preformed creatinine.	Creatine as creatinine.	Dietary additions.
	<i>cc.</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	
5	1,650	3.63	1.012	0.033	
6	1,775	2.48	0.918	0.047	1 gm. thyroid.
7	1,800	2.66	0.990	0.084	1 " "
8	1,800	2.91	1.023	0.324	3 " "
9	1,650	2.57	0.957	0.264	
10	1,450	2.95	1.050	0.058	

## EXPERIMENTAL.

In the following experiments desiccated sheep's thyroid prepared by Armour and Company was given to pigs after their nitrogen metabolism had been reduced to the endogenous type by starch feeding. The sheep's thyroid contained 0.14 per cent of iodine. It was administered, suspended in water, by stomach tube. Analyses for total nitrogen creatine and creatinine were made daily.

The data in Table I show that small amounts of thyroid are ineffective in changing the nitrogen metabolism. Not until 3 gm. were given was the creatine excretion increased. As the preparation contained only 3.6 mg. of creatine per gm. the increase could not be accounted for as being derived from exogenous

sources. It is significant that the total nitrogen and creatinine remained constant as far as our technique allowed us to determine.

Tables II and III show the discrepancy that obtains between the creatine excretion induced by thyroid feeding and the increase in total nitrogen. Creatinine excretion was not increased which may not be so evident from Table III alone but becomes evident when the creatinine level shown here is compared with that shown

TABLE II.

Pig, male, weight 28 kilos. Energy intake as starch, 74 calories per kilo.

Day of starch feeding.	Volume of urine.	Urinary nitrogen.	Preformed creatinine.	Creatine as creatinine.	Dietary additions.
	cc.	gm.	gm.	gm.	
7	1,450	2.37	0.449	0.142	4 gm. thyroid.
8	1,400	2.88	0.488	0.150	
9	1,700	3.29	0.441	0.456	
10	1,200	2.49	0.393	0.376	
11	1,300	2.13	0.421	0.105	

TABLE III.

Pig, male, weight 29 kilos. Energy intake as starch, 70 calories per kilo. This record is a continuation of that shown in Table IV.

Day of starch feeding.	Volume of urine.	Urinary nitrogen.	Preformed creatinine.	Creatine as creatinine.	Dietary additions.
	cc.	gm.	gm.	gm.	
21	1,500	1.65	0.702	0.160	4 gm. thyroid.
22	1,500	1.89	0.727	0.165	
23	1,700	2.89	0.680	0.707	
24	1,550	2.35	0.737	0.707	
25	1,500	2.37	0.630	0.412	
26	1,500	1.50	0.588	0.169	

on the same animal in Table IV. This again emphasizes the marked limitation of the animal's ability to change creatine into creatinine, otherwise with the large increase in creatine to the point where it even exceeds the creatinine excretion, the latter would also have been increased.

While the total nitrogen increase does not appear to be commensurate with the creatine increase, which amounts to a three- to fourfold multiplication when compared with total creatinine, comparisons appear warranted.



In Table II the total creatinine nitrogen ranges approximately from 8 to 11 per cent of the total nitrogen and in Table III from 16 to 22 per cent of the total nitrogen on different days. If these values could be accepted as indicating a relative constancy of relations in the individual it would lend support to our hypotheses that creatinine is formed from creatine, both having a common origin. If on the other hand they are to be considered inconstant it would tend to prove that thyroid medication exerted a special influence on the direction of protein catabolism in relation to the guanidine nucleus. Our data are hardly extensive enough to warrant either conclusion especially as we know that the urinary constituents obey different laws of excretion (13) and therefore changes in nitrogen distribution subsequent to thyroid administration for only 1 day could be expected to reveal little of importance in these guanidine relations.

It is noteworthy that the effect of thyroid feeding did not become evident until the second day. This was usually though not invariably observed as a few records showed a response on the first day.

For the determination of the effect of thyroid feeding on creatinuria when creatine precursors from exogenous sources were available we used pigs which did not show any increase in creatinuria after moderate casein feeding. When this fact had been established with an animal we gave it the thyroid with casein and later thyroid alone. The casein was always given 1 day after the thyroid as our previous results had shown that the thyroid effect was usually delayed 1 day.

The results of these trials are shown in Tables IV, V, and VI. In Table IV, taking the average of 3 days, there were produced 166 mg. more creatine when casein plus thyroid was fed than when thyroid was fed alone. In Table V the increase was 400 mg. daily for 2 days but in Table VI there is a balance of 60 mg. for 3 days in the opposite direction. The latter is too small to have any contradictory significance in our conclusion that thyroid medication may affect the exogenous as well as the endogenous arginine metabolism, yet it does show that the effect is not of such great magnitude that it is always manifested.

TABLE IV.

Pig, male, same individual as used in experiment shown in Table III. Energy intake as starch or its equivalent, 70 calories per kilo.

Day of starch feeding.	Volume of urine.	Urinary nitrogen.	Preformed creatinine.	Creatine as creatinine.	Dietary changes.
	cc.	gm.	gm.	gm.	
4	1,300	1.35	0.658	0.166	105 gm. casein.
5	1,750	1.22	0.653	0.158	
6	1,200	1.92	0.652	0.150	
7	1,250	2.00	0.660	0.150	
8	1,400	1.82	0.693	0.154	
9	1,400	1.96	0.714	0.184	4 gm. thyroid. 105 gm. casein.
10	1,375	5.28	0.630	1.042*	
11	1,550	4.84	0.762	0.928	
12	1,750	2.97	0.751	0.355	
13	1,650	2.44	0.651	0.159	
14	1,650	1.98	0.732	0.200	4 gm. thyroid.
15	1,500	1.65	0.666	0.160	
16	1,500	1.89	0.702	0.165	
17	1,700	2.89	0.727	0.707	
18	1,550	2.35	0.680	0.707	
19	1,500	2.37	0.630	0.412	
20	1,500	1.50	0.588	0.169	

\* Animal had diarrhea, but not severe enough to interfere with the urine analyses.

TABLE V.

Pig, male, weight 36 kilos. Energy intake as starch or its equivalent, 65 calories per kilo.

Day of starch feeding.	Volume of urine.	Urinary nitrogen.	Preformed creatinine.	Creatine as creatinine.	Dietary changes.
	cc.	gm.	gm.	gm.	
6	2,200	2.66	1.180	0.140	210 gm. casein.
7	2,000	2.28	1.080	0.160	
8	1,550	5.02	1.123	0.124	
9	1,950	6.35	1.014	0.132	
10	1,800	3.74	0.990	0.160	
11	2,000	2.66	1.010	0.110	2 gm. thyroid. 210 gm. casein.
12	1,850	10.02	1.063	0.786	
13	2,550	8.21	1.060	0.317	
14	1,900	3.49	0.912	0.085	
15	2,150	2.66	1.130	0.097	
16	2,100	2.31	1.102	0.105	2 gm. thyroid.
17	2,500	2.12	0.900	0.140	
18	2,700	2.22	1.084	0.152	
19	2,000	2.11	1.063	0.140	

TABLE VI.

Pig, male, weight 28 kilos. Energy intake as starch or its equivalent, 68 calories per kilo.

Day of starch feeding.	Volume of urine.	Urinary nitrogen.	Preformed creatinine.	Creatine as creatinine.	Dietary changes.
	<i>cc.</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	
7	1,300	1.50	0.486	0.144	
8	1,500	1.32	0.450	0.145	
9	1,000	2.10	0.450	0.145	105 gm. casein.
10	1,300	2.60	0.437	0.134	
11	1,100	1.82	0.407	0.115	
12	1,350	2.56	0.437	0.128	4 gm. thyroid.
13	1,250	5.20	0.470	0.460	105 gm. casein.
14	1,550	4.06	0.490	0.254	
15	1,500	2.70	0.410	0.178	
16	1,550	2.32	0.428	0.123	
17	1,400	1.93	0.487	0.134	
18	1,450	2.37	0.449	0.142	
19	1,400	2.88	0.488	0.150	4 gm. thyroid.
20	1,700	3.29	0.441	0.456	
21	1,550	3.03	0.485	0.376	
22	1,200	2.49	0.393	0.243	
23	1,300	2.13	0.421	0.105	

## SUMMARY.

The feeding of sheep's thyroid to a pig on a nitrogen-free diet calls forth a marked stimulation of creatine formation. This is accentuated when creatine precursors from exogenous sources are available. It is suggested that creatine formation is primarily dependent upon the balance that obtains between the arginase and oxidative systems whereby arginine is destroyed. On these premises arginine from exogenous sources is not metabolized into creatine in the same proportions as arginine from endogenous sources because this balance varies in different organs. Furthermore, it is suggested that the thyroid principle may be active in causing creatine formation by accelerating the oxidative system of arginine destruction at the expense of the effect of arginase and that in the thyroid mechanism is to be sought the variable responsible for the difference in reaction of men and women to protein feeding.

Creatinuria is looked upon as the result of the accumulation of creatine up to and beyond the threshold of its excretion. Usually this is prevented by the prevalent rate of conversion of creatine into creatinine which appears to be an invariable reaction.

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## THE DISTRIBUTION OF PHOSPHORIC ACID IN THE BLOOD OF NORMAL INFANTS.

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(Received for publication, April 7, 1921.)

As part of an investigation of the factors influencing the occurrence of anemia in infants and young children it was thought desirable to make a study of the various combinations of phosphoric acid in the blood, since, as has been shown,<sup>1</sup> one of these compounds (the organic phosphorus) is a quantitatively important constituent of the red blood corpuscles, while another (the lecithin) also present in fairly large amounts, is commonly believed to predispose to the destruction of these cells. The work was further desirable as a contribution to the comparative physiology of these compounds which is being investigated in this laboratory for the purpose of getting information regarding their functions, and especially that of the unknown organic phosphorus compound present in large amounts in the corpuscles.

The present report consists of the results of an examination of the blood of normal infants from birth up to 2 weeks of age. For the determination 15 cc. of blood were drawn from the superior sagittal sinus, prevented from clotting by the addition of minimal amounts of oxalate or citrate, and delivered at the laboratory as soon as possible afterwards—generally in 2 to 3 hours. It was then centrifuged in graduated centrifuge tubes for 10 minutes at about 4,000 R. P. M. and the levels of total blood and corpuscles read off for the purpose of determining the ratio of corpuscles to plasma. Hemolyzed blood was rejected because of the probability that significant amounts of corpuscle phosphorus had passed into the plasma. After separation of the plasma from the corpuscles, determinations of the various phosphoric acid compounds were made in each, using the methods previously described.<sup>2</sup>

The results of the examination are given in Table I.

<sup>1</sup> Bloor, W. R., *J. Biol. Chem.*, 1918, xxxvi, 49.

<sup>2</sup> Bloor, W. R., *J. Biol. Chem.*, 1918, xxxvi, 33.

TABLE I  
Phosphoric Acid Compounds in the Blood of Infants.

Case No.	Name	Age, days	Corp. ele., per cent	Change in weight, gm.	Plasma.				Corpuscles				H <sub>2</sub> PO <sub>4</sub> per 100 cc.	
					Total.	Acid-soluble.	In-organic.	Lipoid.	Or-ganic.	Total.	Acid-soluble.	In-organic.	Lipoid.	Organic
					mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.
1	Boys. C. K.	10		3,840	29.3	13.2		16.6		208.2	137.0	14.7	62.5	122.3
				3,485										
3	M. A.	26	24.0	3,595 3,635	21.7	10.0	6.6	11.5	3.4	284.0	214.0	9.4	59.5	204.5
4	N. M.	13	40.5	3,490 3,460	18.5	8.8	7.5	10.7	1.3	281.0	221.0	10.8	59.5	210.2
6	S. N.	2	45.0	3,330 3,200	23.4	13.2	7.7	10.1	5.5	260.0	178.0	13.5	74.8	154.5
9	T. S.	5	51.7	5,970 5,900						234.0	175.0	26.7	52.0	148.3
13	M. N.	7	43.2	3,270 3,180	35.0	23.6	9.6	18.2	14.0	275.7	216.0		58.0	
14	M. N.	6	41.9	3,080 3,010	34.0	18.0	10.2	15.9	7.8	240.3	190.0		47.6	

15	M. A.....	6	53.1	2,400 2,460	31.5	18.0	9.1	13.8	8.9	250.0	175.0	49.0	
17	P. Y.....	11		3,000 3,050	30.0		10.7	17.2		240.0	195.3	46.8	
18	H. Y.....	12	49.0	3,430 3,345	35.9	14.5	10.7	21.4	3.8	260.4	204.0	56.6	182.1
Average .....			43.5		28.8	14.9	9.0	15.0	6.4	253.4	190.5	56.6	170.3
Girls.													
2	J. N.....	12	40.5	3,370 3,370	22.5	12.8		11.1		347.0*	135.0	73.0	121.5
5	V. R.....	15	40.5	3,450 3,330	23.9	13.6	8.0	10.4	5.6	221.0	151.0	60.0	136.8
8	S. Y....	10	51.3	2,810 2,720						253.0	200.0	54.3	178.6
10	R. D.....	15	51.0	3,360 3,180	45.0*	25.0	13.9	17.3	11.1	240.0	187.0	53.2	176.6
12	F. S.....	5	43.0	4,690 4,820	35.0	16.3	10.0	20.8	6.3	279.0	219.7	58.1	
16	D. E.....	13	52.0	3,470 3,340	35.0		12.5	13.0		234.0	163.0	35.7	

\* Not included in average.

TABLE I *Continued.*

Case No.	Name	Age, days	Cupus, per cent	Chlorophyll, gm.	Plasma			H <sub>2</sub> PO <sub>4</sub> per 100 cc.					Corpuscles		
					Total	Acid-soluble	In-organic	Lipoid	Or- ganic	Total	Acid-soluble	In-organic	Lipoid	Or- ganic	Total
					mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.
19	L. Z.	11	36.4	2,670 2,905	29.4	17.5	12.9	16.6	4.8	208.0	151.2	12.8	44.1	138.4	
20	L. L.		37.2	2,670 2,765	43.1	25.0	11.6	17.5	11.4	231.3	173.6	15.1	50.0	158.5	
21	W. N.	17	45.2	4,120 4,065	21.5	15.7	10.9	12.9	4.6	186.5	142.0	12.5	42.7	129.5	
22	O. N.	2	45.0	2,620 2,605	24.0	17.5	11.3	10.6	5.3	198.4	143.0	13.8	55.5	130.2	
23	O. N.	3	51.0	3,815 3,815	23.4	12.2	10.0	11.6	2.2	260.0	175.5	17.0	45.5	158.5	
Average			45.1		30.3	17.4	11.2	14.1	6.4	231.4	167.3	14.9	52.0	147.6	



## DISCUSSION.

*Influence of Sex.*—Very little difference can be noted in the values as characteristic of the sexes. In the plasma the values for inorganic phosphorus are markedly lower in the boys than in the girls while the lipid phosphorus is slightly higher but not significantly so. The values for organic phosphorus are the same in both and show the same wide variations. In the corpuscles the only striking difference is in the organic phosphorus which is considerably higher in the boys. Since, however, relatively few determinations are available much emphasis cannot be laid on these differences.

TABLE II.  
*Averages and Variations in the Phosphoric Acid Compounds in the Blood of Infants and of Adults.*

		Plasma.					Corpuscles				
		Total.	Acid-soluble.	Inorganic	Lipoid.	Organic.	Total.	Acid-soluble.	Inorganic.	Lipoid.	Organic.
Low	Adult.....	27.6	8.4	7.0	17.5	0.2	200.0	150.0	10.9	45.0	128.0
	Infant....	18.5	8.8	3.7	10.1	1.3	186.5	135.0	9.4	42.7	121.5
Average	Adult.....	34.0	11.4	9.9	23.5	1.5	248.0	187.0	17.2	57.0	169.5
	Infant....	29.7	16.5	9.6	14.8	7.0	241.4	179.6	14.3	56.0	156.0
High	Adult.....	42.2	14	14	26.2	4.0	295.0	228.0	26.8	64.0	213.0
	Infant....	43.0	25	13.9	21.4	11.4	284.0	221.0	26.7	74.8	204.5

*Influence of Weight Changes.*—Most of the infants were losing weight at the time the samples were taken so that little can be said with regard to the effect of gaining or losing weight on the blood phosphates. As far as can be seen, however, in infants gaining weight the values are higher than the average in the plasma and somewhat lower in the corpuscles, while in those losing weight the reverse is the case although the difference is less noticeable.

*Comparison with Adults.*—For convenience in comparison there are given in Table II the averages and variations in the phosphoric acid compounds of infants and adults.

*Corpuscles.*—The average values for total and lipid phosphorus in the corpuscles of adult and infant agree very closely while the average inorganic value is considerably, and the organic somewhat, lower in the infant. The low values are lower in the infant than in the adult as is also the case with the high values with the exception of the lipid phosphorus which reaches higher levels than are normally found in the adult. The latter may be due to the almost continuous absorption of fat in infants of this age, since high values for fat in blood have been found to bring about increases of lipid phosphorus in the corpuscles.

The corpuscle values as a whole are remarkably similar in the infant and the adult and indicate that there is little if any change in the composition of the red cells after birth—a finding which is in marked contrast to that in cattle in which Meigs<sup>3</sup> found much higher values (nearly twice in some cases) for total phosphorus in the corpuscles of young calves than in those of cows.

*Plasma.*—The organic phosphorus is regularly much higher in the infant than in the adult, resulting also in a higher acid-soluble fraction. Lipid phosphorus is much lower throughout in the infant than in the adult, in this respect agreeing with the findings of Meigs<sup>3</sup> with cattle in which the lipid phosphorus of the plasma of calves is found very low, increasing up to the age of about 1 year.

<sup>3</sup> Meigs, E. B., Blatherwick, N. R., and Cary, C. A., *J. Biol. Chem.*, 1919, xxxvii, 1.

# THE DETERMINATION OF INORGANIC SULFATE, TOTAL SULFATE, AND TOTAL SULFUR IN URINE BY THE BENZIDINE METHOD.

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(Received for publication, April 28, 1921.)

As far as its use for the analysis of urine is concerned, the benzidine method for the determination of sulfur is subject to two sources of error that may under certain conditions be serious. One of these, the contamination of the benzidine sulfate precipitate by phosphate, has been recognized before, and can be eliminated by adding acid before precipitating with benzidine. For this purpose Rosenheim and Drummond<sup>1</sup> recommend the addition of hydrochloric acid to the urine until Congo red paper shows an acid reaction. Gauvin and Skarzynski,<sup>2</sup> on the other hand, add the same quantity of hydrochloric acid in every instance, without the use of an indicator, and this scheme has also been adopted by Drummond<sup>3</sup> in a more recent modification of the Rosenheim and Drummond method on a smaller scale. In the methods for inorganic sulfate proposed by all these writers, the concentration of benzidine during the precipitation is about the same ( 0.017 to 0.02 N ), but the concentration of hydrochloric acid is much less uniform. In Table I are collected data showing the concentrations of sulfuric acid, benzidine, and hydrochloric acid existing during the precipitation from sulfate solutions by these three methods. The figure for hydrochloric acid given in the table for Rosenheim and Drummond's method holds also with urine, since in this method the urine is acidified before adding the benzidine reagent. In the other two methods, in-

<sup>1</sup> Rosenheim, O., and Drummond, J. C., *Biochem. J.*, 1914-15, viii, 143.

<sup>2</sup> Gauvin, R., and Skarzynski, V., *Bull. Soc. chim.*, 1913, 4th series, xiii, 1121.

<sup>3</sup> Drummond, J. C., *Biochem. J.*, 1915, ix, 492.

asmuch as this step is omitted, the concentration of hydrochloric acid during the precipitation of benzidine sulfate from urine is less than stated by an amount that varies with the character of the urine.

In spite of these differences, all three methods have given results that agree fairly well with the gravimetric method, but they have done so only because the comparisons have been confined to urines that are very much alike with respect to the factors that affect the accuracy of the determination. As long as their use is restricted to ordinary 24 hour urines, any one of a great many possible modifications would suffice to avoid trouble due to the presence of phosphate, simply because in such urines there is never a very large amount of phosphate in proportion to the sulfate content. But the much more exacting requirements of

TABLE I.

Method.	Sulfuric acid.	Benzidine.	Hydrochloric acid.
	N	N	N
Rosenheim and Drummond.....	0.004-0.007	0.017	0.024
Drummond.....	0.003-0.010	0.017	0.084
Gauvin and Skarzynski.....	0.002-0.003	0.020	0.031

the less uniform urines obtained in short period metabolism experiments are satisfactorily met neither by any modification of the benzidine method that has been suggested so far, nor by any that I have been able to find that does not involve the preliminary removal of the phosphate.

If phosphate were the only source of trouble, it would be easy to devise a method that would do for all circumstances that are likely to arise, although such a method would call for a more precise adjustment of the acidity than has heretofore been thought necessary. But unfortunately there is a second cause of error, in some respects even more troublesome. This is the increase in solubility of benzidine sulfate due to the presence of chloride, which often exists in urine in sufficient concentration to be injurious in this way. That this may rarely or never be the case in 24 hour urines presumably accounts for the fact that the effect of chloride has previously escaped notice in connection with urine analysis.

In 1 hour urines, on the other hand, for each mg. of sulfur in the form of inorganic sulfate, there may be as much as 5 mg. of inorganic phosphorus and 30 mg. of chlorine, and even more under conditions that can hardly be called unusual, although they may not be especially frequent. The influence of the addition of sodium chloride and disodium phosphate on the analysis of sulfate solutions by Drummond's method will be seen from the figures recorded in Table II. That the same factors

TABLE II.

*Analysis of Sodium Sulfate Solution by Drummond's Method.*

Composition of solution.			Sulfur found.
Sulfur.	Phosphorus.	Chlorine.	
mg.	mg.	mg.	mg.
1	0	0	1.00
1	5	0	1.04
1	0	30	0.97
1	0	60	0.91

TABLE III.

*Analysis of Urine by Drummond's Method.*

Experiment No.	Urine.	Inorganic phosphorus.	Chlorine.	Inorganic sulfate sulfur found.	
		mg.	mg.	mg.	mg. per 100 cc.
1	1	0.66	14.8	0.674	67.4
2	1	3.36	14.8	0.678	67.8
3	1	0.66	34.8	0.642	64.2
4	2	1.32	29.6	1.39	69.5
5	2	6.72	29.6	1.44	72.0

are not without considerable effect on sulfate determinations in urine is evident from the results in Table III, all obtained with one sample of urine, which was analyzed alone (Experiments 1 and 4) and after the addition of disodium phosphate (Experiments 2 and 5) and of sodium chloride (Experiment 3). Depending upon the amount of phosphate and chloride present (and the conditions in this respect are not extreme for 1 hour periods) the figures vary by more than 10 per cent.

A considerable experience with short period metabolism experiments has led me to the conclusion that any sulfur method,

to be safe for such work, must give accurate results in the presence of 10 mg. of phosphorus or 60 mg. of chlorine for each mg. of sulfur in the form of inorganic sulfate. Many different benzidine reagents have been tried, under all sorts of conditions, but none has been found equal to these requirements, and it is apparently necessary to accept the fact that both these difficulties cannot be successfully contended with at the same time. Once the phosphate has been removed, the situation is much simplified, as it is then possible to avoid trouble from the presence of chloride by precipitating the benzidine sulfate at a much lower acidity than would otherwise be permissible.

#### *Removal of Phosphate.*

For the removal of phosphate from urine as a preliminary to the precipitation of sulfate with benzidine, nothing has been found equal in effectiveness to magnesia mixture in some form, and it is fortunately possible to precipitate the phosphate nearly quantitatively as magnesium ammonium phosphate without the introduction of injurious quantities of electrolytes, which, like sodium chloride, would prevent the complete precipitation of benzidine sulfate. This is accomplished by shaking the urine (previously neutralized with ammonia) with basic magnesium carbonate in the presence of a small amount of ammonium chloride. The whole process of precipitation and filtration requires only a very few minutes. If the first 15 or 20 cc. of filtrate are poured back on the paper and filtered again, the solution will percolate through a layer of magnesium carbonate mixed with triple phosphate crystals, and this is a particularly effective way of removing phosphate. The final filtrate should then contain less than 0.1 mg. of inorganic phosphorus in 5 cc.

The urine must be fairly dilute before the phosphate is removed, for magnesium ammonium phosphate crystallizes with 8 molecules of water, and the removal in this way of more than 0.2 per cent of phosphorus would appreciably alter the concentration of sulfate in the filtrate.

In the following directions for preparing the essentially phosphate-free filtrate, the quantities prescribed are sufficient for duplicate determinations of all three forms of sulfur (inorganic sulfate, total sulfate, and total sulfur).

Transfer to a 50 cc. volumetric flask sufficient urine to contain between 5 and 10 mg. of sulfur in the form of inorganic sulfate, and dilute to about 25 cc. with water. Add 1 drop of phenolphthalein solution and 1 drop of concentrated ammonium hydroxide (or as much as is necessary to make the solution faintly pink), followed by 5 cc. of a 5 per cent solution of ammonium chloride. Make up to the mark, mix, and pour the solution into a dry Erlenmeyer flask containing about 0.65 gm. of *finely powdered* basic magnesium carbonate.<sup>4</sup> Shake for 1 minute, and transfer to a 9 cm. filter paper enough of the suspension to fill the paper nearly to the top. Allow this first filtrate to drain back into the Erlenmeyer flask, and then filter the entire suspension through the same paper into a dry container.

In case the urine is already extremely dilute, the phosphate can be precipitated without appreciably altering the concentration by using solid ammonium chloride (0.25 gm.) instead of a solution. Urines obtained in short period experiments are sometimes so dilute as to make this modification necessary.

The filtrate, prepared as described above, is now used for all three sulfur determinations.

#### *Determination of Sulfur in the Phosphate-Free Filtrate.*

*Inorganic Sulfate.*—Pipette 5 cc. of the filtrate into a 100 cc. beaker. Add 2 drops of a 0.04 per cent alcoholic solution of brom-phenol blue<sup>5</sup> and 5 cc. of water. Then add approximately  $N$  HCl, drop by drop, until the solution is yellow without a trace of blue. Run in, from a pipette, 2 cc. of benzdine reagent,<sup>6</sup> and let stand for 2 minutes. Finally, add 4 cc. of 95 per cent acetone, and let stand for 10 minutes more. Filter through a mat of paper pulp in a special filtration tube (described below). Wash the beaker and the filter, first with three 1 cc. portions of 95 per cent acetone, and then once with 5 cc. Transfer about 2 cc. of water to the filtration tube, and poke the precipitate

<sup>4</sup> This reagent must obviously be free from sulfate. Baker's analyzed magnesium carbonate has proved satisfactory.

<sup>5</sup> Clark, W. M., The determination of hydrogen ions, Baltimore, 1920, 63.

<sup>6</sup> Suspend 4 gm. of benzdine in about 150 cc. of water in a 250 cc. volumetric flask. Add 50 cc. of  $N$  HCl (standardized). Shake until dissolved, and make up to volume. Filter if necessary.

and mat through the hole in the lower end into a large Pyrex test-tube (200 by 20 mm.), using a sharpened nichrome wire. Rinse off the wire with a few drops of water, and heat the contents of the test-tube just to boiling, leaving the filtration tube suspended in the mouth of the test-tube. Add 2 drops of a 0.05 per cent aqueous solution of phenol red (monosodium salt),<sup>5</sup> and run in from a micro-burette,<sup>7,8</sup> through the filtration tube, about 1 cc. of 0.02  $\times$  NaOH. Rinse down the wall of the filtration tube with 2 or 3 cc. of water from a wash bottle, heat again to boiling until steam escapes actively from the test-tube, and rinse a second time with sufficient water to bring the total volume up to about 10 cc. This treatment should suffice to remove all traces of precipitate from the filtration tube, which may now be removed, and the titration with 0.02  $\times$  NaOH continued. When the color begins to change from yellow to red, again heat to boiling, and pour the hot solution into the beaker (in which the precipitation took place) and back.<sup>9</sup> This will decompose any trace of precipitate that may have adhered to the wall of the beaker. From this point on the standard alkali should be added, not more than 0.02 cc. at a time, until the solution acquires a definite pink color, which further boiling does not discharge.

*Total Sulfate.*—To 5 cc. of the filtrate in a 100 cc. beaker add 1 cc. of 3  $\times$  HCl (approximate). Heat on the water bath until the solution has evaporated to dryness, and for 10 minutes longer. Immediately add 10 cc. of water, and break up the residue by rotating the beaker. Add 2 cc. of the benzidine reagent and (2 minutes later) 4 cc. of acetone, exactly as in the method for inorganic sulfate, and complete the determination as described above.

*Total Sulfur.*—Transfer 0.25 cc. of Benedict's total sulfur reagent<sup>10</sup> to a 6 cm. evaporating dish, and add 5 cc. of the urine

<sup>7</sup> Folin, O., and Peck, E. C., *J. Biol. Chem.*, 1919, xxxviii, 289.

<sup>8</sup> Fiske, C. H., *J. Biol. Chem.*, 1921, xlv, 285.

<sup>9</sup> This step may be avoided by conducting both precipitation and titration in a large lipped test-tube, but except for the inorganic sulfate determination a beaker is on the whole more convenient.

<sup>10</sup> Benedict, S. R., *J. Biol. Chem.*, 1909, vi, 363. The reagent contains 20 gm. of copper nitrate crystals and 5 gm. of potassium chlorate per 100 cc. A blank (gravimetric) must, of course, be run on the reagent unless the copper nitrate is free from sulfate.



filtrate. Evaporate to dryness, preferably on an electric hot plate at low heat. When the mixture has become dry, increase the heat by steps to the maximum, and finish the ignition with a microburner, allowing 2 minutes at red heat after the contents of the dish have become thoroughly black. Cool for 5 minutes. Add 1 cc. of 3 N HCl, and evaporate to dryness on the hot plate (low heat). When the residue is thoroughly dry, dissolve and wash into a 100 cc. beaker with five 2 cc. portions of water. Add 1 drop of N HCl, and precipitate with the benzidine reagent and acetone as in the other two methods. The rest of the determination is likewise the same as before, with the single exception that 2 cc. of 50 per cent acetone should be used in place of the first of the three 1 cc. portions of 95 per cent acetone, otherwise it will be impossible to wash the filter free from copper.

The amount of sulfur in the 5 cc. of filtrate analyzed is in each case obtained (in mg.) by multiplying the titration figure by 0.32.

#### DISCUSSION.

*Use of Acetone.*—At a time when there was still some hope of avoiding the necessity of removing the phosphate, the addition of acetone during the precipitation with benzidine was introduced for the purpose of diminishing the solubility of the precipitate. Although this may not be altogether necessary under the conditions finally adopted, the modification has been retained, and the same liquid used for washing the precipitate, for various reasons. Washing with a saturated solution of the precipitate is a thing to be avoided whenever possible, and acetone is to be preferred on that account. Since acetone wets glass more readily than does water, it does not collect in drops on the wall of the beaker, a matter of some consequence when a fairly large surface must be washed with a small volume of liquid. But the most important consideration of all is that the use of acetone altogether prevents the benzidine sulfate from assuming the form of large flakes, which can be decomposed only by prolonged boiling at the end of the titration, and on account of which Rosenheim and Drummond,<sup>1</sup> who wash the precipitate with a saturated solution of benzidine sulfate, have been obliged to recommend that the filter never be allowed to be sucked dry.

*Indicator.*—Boiling aqueous solutions of purified benzidine, at concentrations corresponding with the conditions at the end-point of the titration described above, give a brownish color with phenol red, intermediate between yellow and red. The addition of less than 1 per cent of one equivalent of sodium hydroxide is sufficient to change the color to a definite pink, whereas phenolphthalein under these conditions is still colorless. Since phenol red is besides a much more brilliant indicator, it is to be preferred on all counts.

*Filtration Tube.*—This is, in principle, the same as the tube recently described in connection with a method for the determination of inorganic phosphate in urine.<sup>8</sup> The narrow tube recommended for the filtration of magnesium ammonium phosphate

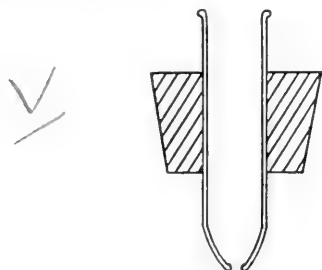


FIG. 1. Filtration tube (one-half natural size).

is readily clogged by the much more compact benzidine sulfate precipitate, and for sulfate determinations it should be replaced by one considerably larger in diameter. The tubes that have been found most convenient are made from glass tubing<sup>11</sup> 15 mm. in internal diameter, shrunk at one end so as to leave a hole 3 mm. in diameter, cut to a length of 70 mm., and flanged at the cut end. The somewhat elongated tip shown in Fig. 1 gives the best results.

In using this tube, only enough paper pulp should be introduced to form a thin cup-shaped mat lining the constricted tip. It is neither necessary nor desirable to fill the tip with pulp. The mat having been prepared, the tube should be filled with the

<sup>11</sup> Pyrex tubing is preferable. Since the tube is subjected to the action of steam during the titration, soft glass should not be used.

solution to be filtered *before* starting the suction, and the suction, when it is started, should be very gentle. Any attempt to hasten the filtration by applying strong suction is almost certain to produce the opposite result by packing down the precipitate.

*Adjustment of Acidity.*—The purpose of the preliminary acidification ( to brom-phenol blue ) in the determination of inorganic sulfate is to neutralize any basic substances that may be present and to liberate weak acids from their salts. No appreciable

TABLE IV.

Urine No.		Inorganic sulfate S.  <i>mg. per 100 cc.</i>	Inorganic P Inorganic sulfate S	Chlorine Inorganic sulfate S	Sulfur per hr.					
					Inorganic sulfate.		Total sulfate.		Total sulfur.	
					Gravi- metric.	Titra- tion.	Gravi- metric.	Titra- tion.	Gravi- metric.	Titra- tion.
					<i>mg.</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>
1	(a)	21.0	1.8	14	15.0	15.4	17.4	17.3	21.1	21.1
	(b)		10.0	14		15.4		17.3		20.9
	(c)		1.8	60		15.3		17.3		21.1
2	(a)	9.5	0.9	13	22.7	22.6	25.4	25.6	32.6	32.5
	(b)		10.0	13		22.8		25.6		
	(c)		0.9	60		22.4		25.3		32.3
3	(a)	8.5	1.0	18	14.3	14.8	17.7	17.8	22.1	22.4
	(b)		10.0	18		14.7		17.9		22.3
	(c)		1.0	60		14.7		18.0		22.3
4	(a)	20.5	0.8	9	34.5	34.7	37.5	37.7	42.0	41.7
	(b)		10.0	9		34.8		37.7		41.8
	(c)		0.8	60		34.3		37.7		42.2

amount of the hydrochloric acid in the benzidine reagent will then be neutralized by constituents of the urine filtrate. In the method for total sulfate, this adjustment is made automatically by evaporating the urine with hydrochloric acid.

*Hydrolysis of Ethereal Sulfate.*—Evaporation with hydrochloric acid is substituted for the usual boiling mainly because the additional acid required for hydrolysis would be enough to cause low results in the presence of large amounts of chloride.

*Total Sulfur Method.*—The only special modification introduced in this determination is the removal of the excess hydrochloric acid, after dissolving the residue of copper oxide, by evaporation to dryness instead of by neutralization with alkali. Neutralization would introduce more sodium chloride, which is undesirable for reasons that have been mentioned. After the excess acid has been removed by evaporation, the residue is at times so nearly neutral that a drop of dilute acid should be added (as stated) before running in the benzidine reagent.

### *Results.*

The method as described gives satisfactory results in the presence of 10 mg. of inorganic phosphorus or 60 mg. of chlorine for each mg. of sulfur in the form of inorganic sulfate. A series of analyses is given in Table IV. Each of the urines was analyzed first in the manner described above (*a*), secondly after adding sufficient disodium phosphate to bring the inorganic phosphate content up to the stated figure (*b*), and finally after the addition of enough sodium chloride to make the ratio of chlorine to inorganic sulfate S equal to 60 (*c*). For comparison the same urines were analyzed gravimetrically (Folin's<sup>12</sup> method for inorganic and total sulfate; Benedict's<sup>10</sup> method for total sulfur).

Differences greater than about 1 per cent sometimes occur in the determination of inorganic sulfate, especially when the sulfur excretion is small, however closely duplicates by each method may agree. In such cases there is apparently no way of deciding which method is the more accurate. Discrepancies of this nature have not been observed in the determination of total sulfate or total sulfur.

<sup>12</sup> Folin, O., *J. Biol. Chem.*, 1905, i, 131.

## BASAL METABOLISM OF NORMAL WOMEN.\*

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(Received for publication, April 9, 1921.)

The primary purpose of this investigation was to find out whether there is any regular periodic variation in the basal metabolism of normal women during menstruation. The figures obtained show the lack of such periodicity. They bring out also the marked day by day variation in basal metabolism which may be expected at any time, and the 216 observations add to the already accumulated data on standards for women. These three points will be taken up successively.

### *Procedure for Determining Metabolism.*

#### *Method.*

The Benedict portable respiration apparatus was used to determine the basal metabolism. In general, the procedure of the Nutrition Laboratory for determining oxygen consumption was followed (1, 2). The subject, in a postadsorptive condition, took a 30 minute rest period in a quiet room preceding the two 10 minute observations. The initial reading for the volume of oxygen was taken after the subject was connected with the air current at the end of a series of regular expirations, and at the same instant a stop-watch was started. With a second stop-watch another reading was taken so that duplicate records were made for each 10 minute period. The day's basal metabolism was taken as the average of the duplicates of the two 10 minute periods. If for any subject checks were not obtained on the first two periods, a third period was observed. Very occasionally

\* The work reported in this paper will form part of the thesis to be submitted by Marie Dye for the degree of Doctor of Philosophy, University of Chicago.

a whole day had to be discarded because of failure to obtain satisfactory duplicates. The results are given only when duplicate readings agree within 5 per cent. No attempt was made to determine the carbon dioxide. Benedict (1) has found that the error, made in assuming a constant respiratory quotient, when the subject has been 12 or more hours without food, is slight. We have assumed the customary respiratory quotient of 0.82 and the factor 4.825 calories per liter of oxygen.

*Subjects.*—Our seventeen subjects were faculty members or students at the University of Chicago from 21 to 44 years old. All were in normal physical condition, and continued their usual academic work during the menses, four of them stating that they felt absolutely no discomfort or disturbance of any kind (Nos. 12, 13, 14, and 15) and the others experiencing more or less fatigue or lassitude. From one to three complete periods were studied with pre- and intermenstrual observations on each of fourteen of the subjects. One case of amenorrhea (No.1) was observed for 26 almost successive days. Two intermenstrual studies of 10 days each were made (Nos. 3 and 8).

#### *Experimental Data.*

The detailed results of all observations are given in Table VI, listing the subjects according to age. The age is that at the nearest birthday, the weight is taken nude, and the surface area is read from the DuBois height-weight chart (3).

#### *Comparison of Metabolism during Menstrual and Intermenstrual Phases.*

*Averages for Menstrual and Intermenstrual Phases.*—In Table I are brought together the averages of all the observations made on the fourteen women during the menstrual and the intermenstrual periods. The figures show no consistent variation. In only six of the subjects is the difference between averages for the two phases greater than 3 per cent, and of these six, some have the higher metabolism during menstruation and some in the intermediate period. The average of all the subjects gives a 1.6 per cent lower metabolism during menstruation than at other times and practically the same decrease when only the

first 2 menstrual days are included. This difference is too small to be considered significant.

*Periodicity of Metabolism during Premenstrual and Menstrual Phases.*—There also seems to be no regular, rhythmic, day by day variation. The curve of the daily observations is far from smooth. Table II, which lists the daily metabolism immediately before and during menstruation, gives a confused sense of

TABLE I.

*Comparison of Average Heat Production during Menstrual and Intermenstrual Phases.*

Subject.	Age.	Height.	Weight.	Surface area.	Inter-menstrual.		Menstrual.			First 2 menstrual days.		
					Days observed.	Calories per 24 hrs.	Days observed.	Calories per 24 hrs.	Above inter-menstrual.	Days observed.	Calories per 24 hrs.	Above inter-menstrual.
		cm.	kg.	sq. m.	No.		No.		per cent	No.		per cent
2	24	152	66.7	1.63	3	1,407	6	1,445	2.7	2	1,430	1.6
4	25	164	64.1	1.70	2	1,277	3	1,332	4.3	2	1,357	6.3
5	26	161	50.1	1.51	5	1,281	11	1,231	-3.9	6	1,229	-4.0
6	28	148	53.0	1.44	4	1,137	8	1,132	-0.4	3	1,170	2.9
7	28	160	61.0	1.63	3	1,353	6	1,318	-2.6	4	1,322	-2.3
9	29	149	51.0	1.43	3	1,238	6	1,272	2.7	4	1,281	3.5
10	30	170	61.7	1.70	2	1,537	7	1,468	-4.5	3	1,505	-2.1
11	32	151	43.0	1.34	3	1,135	3	1,068	-5.9	2	1,052	-7.3
12	33	168	62.0	1.69	2	1,360	3	1,337	-1.7	2	1,315	-3.3
13	33	173	79.0	1.93	3	1,633	8	1,551	-5.0	2	1,562	-4.3
14	37	162	75.5	1.80	2	1,500	5	1,454	-3.1	4	1,454	-3.1
15	40	166	66.6	1.74	2	1,510	3	1,437	-4.8	2	1,442	-4.5
16	41	158	70.0	1.71	2	1,347	4	1,329	-1.3	2	1,310	-2.7
17	44	162	49.1	1.49	7	1,203	11	1,224	1.7	5	1,210	0.5
Average.						1,351		1,328	-1.6		1,331	-1.3

irregularity rather than of periodicity. With a number of subjects a high metabolism day can be found just before or at the beginning of menstruation, but so also can a low metabolism day. Moreover, the high day connected with menstruation is only sometimes higher than other days observed. The only possible conclusion seems to be that the variation connected with the menstrual cycle is irregular and no greater than at other times.

We have been able to find in the literature only three other series of observations on basal metabolism during menstruation, a long series by Zuntz (4) in 1906, two observations by Gephart and Du Bois (5) in 1916, and a preliminary report by Ford in a

TABLE II.

*Daily Variations during Premenstrual and Menstrual Phases in Calories per 24 Hours.*

Subject.	2 days before menstruation.	Day before menstruation.	Day of menstruation.					
			1st	2nd	3rd	4th	5th	6th
2	1,390*	1,355	1,500	1,360	1,440	1,440	1,440	1,490
4	1,375*		1,355	1,360	1,280			
5 (I)	1,375*	1,375	1,300	1,390	1,330	1,230		
5 (II)		1,370	1,235	1,195	1,195	1,330		
5 (III)	1,100	1,135	1,110	1,145	1,080			
6 (I)			1,200		1,090	1,070	1,125	
6 (II)	1,200	1,145	1,210	1,100	1,140	1,125		
7 (I)			1,390	1,310	1,310			
7 (II)	1,320	1,320	1,300	1,290	1,310			
9 (I)		1,250	1,310	1,260	1,270			
9 (II)		1,230	1,265	1,290	1,235			
10 (I)	1,540	1,515	1,490	1,425	1,465	1,475		
10 (II)	1,585	1,425	1,600		1,420	1,400		
11	1,125	1,105	1,085	1,020	1,105			
12			1,320	1,310	1,380			
13 (I)		1,650	1,620	1,520	1,620	1,515		
13 (II)		1,585	1,575	1,535	1,535	1,490		
14 (I)		1,390	1,455	1,390	1,455			
14 (II)		1,460	1,480	1,490				
15	1,410		1,425	1,460	1,425			
16	1,290	1,360	1,370	1,250	1,320	1,375		
17 (I)		1,145	1,315		1,270	1,300		
17 (II)		1,080	1,140	1,140		1,220	1,235	
17 (III)	1,260	1,190	1,235	1,220	1,220	1,175		

\* The actual observations of cc. of oxygen per minute from which these figures were calculated were for No. 1: 202, 202, 200, and 197; for No. 2: 193, 198, 200, and 200; and for No. 3: 197, 198, 200, and 199. These may be taken as typical of the range in our duplicate observations.

recent paper by Snell, Ford, and Rowntree (6). Zuntz determined the carbon dioxide output and part of the time the oxygen consumption for two women for 97 days almost without a break, including three menstrual periods with inter-, pre-, and



postmenstrual observations. His conclusions, and also those of Gephart and Du Bois, are in line with ours—that there is no regularity of variation.

Snell, Ford, and Rowntree, on the other hand, conclude that menstruation “does affect the basal metabolic rate of women at times in health and disease.” Ford concluded for eight cases out of ten studied that a rather constant rise occurs during menstruation or in the premenstrual period, the rise being followed by a postmenstrual fall. Two of the ten cases showed a drop rather than a rise. The details of this work have not yet been published. We have no information of our subjects in regard to postmenstrual metabolism, but we do not find regularly the menstrual or premenstrual rise that Ford does.

#### *Daily Variation in Metabolism.*

As already pointed out in the discussion of menstrual variation, the extremes of metabolism of these subjects on different days show a wide range. Since this is as marked during the intermenstrual as the premenstrual and menstrual phases, no distinctions of phases are made in this part of the discussion. The maximum and the minimum metabolism observed at any time for each subject, including the three subjects observed without menstrual phase, are brought together in Table III. The percentage of the maximum above the minimum is very high in many cases. The highest difference is 28.8 per cent, the lowest 7.4 per cent, and the average 13.2 per cent. Only six of the seventeen subjects show a range lower than 10 per cent which the clinician often uses as his criterion of normality as compared with a standard. Even if the variations are calculated from the average for the individual, instead of by comparison of maximum and minimum values, three of the subjects still have a greater range than 10 per cent.<sup>1</sup> Our observations, therefore, emphasize the importance of taking several days' rather than

<sup>1</sup> Compare the recommendation of Boothby (7) in a recent article: “all patients having a rate ranging between +10 and +20 per cent. should have the test repeated on a subsequent day.”

a single day's observation to arrive at what is average for an individual.<sup>2</sup>

These observations on the high variation which may be expected for an individual on different days merely confirm those given by Benedict in 1915 (8). At that time he listed the ex-

TABLE III.  
*Extreme Variations.*

Subject	Total No. of days observed.	Heat production per day.			Variation of maximum above minimum.		Extreme variation from average.	
		Maximum.	Minimum.	Average.	calories	per cent	calories	per cent
1	26	1,320	1,125	1,239	195	17.3	-114	-9.2
2	14	1,500	1,355	1,413	145	10.7	87	6.2
3	10	1,345	1,125	1,205	220	19.6	140	11.6
4	6	1,375	1,260	1,321	115	9.1	-61	-4.6
5	25	1,390	1,080	1,254	310	28.8	-174	-14.0
6	14	1,210	1,070	1,140	140	13.1	-70	-6.1
7	13	1,390	1,290	1,335	100	7.8	55	4.1
8	10	1,495	1,340	1,396	155	11.6	99	7.1
9	11	1,310	1,220	1,256	90	7.4	54	4.3
10	13	1,600	1,400	1,493	200	14.2	107	7.2
11	8	1,170	1,020	1,105	150	14.7	-85	-7.7
12	5	1,430	1,290	1,346	140	10.8	84	6.2
13	13	1,660	1,490	1,580	170	11.4	-90	-5.7
14	9	1,510	1,390	1,457	120	8.6	-67	-4.6
15	7	1,520	1,410	1,457	110	7.8	63	4.3
16	9	1,375	1,250	1,331	115	9.5	-81	-6.1
17	29	1,315	1,080	1,218	235	21.8	-138	-11.3
Average.						13.2		

treme variations for a large number of his subjects who had been studied 5 days or more. His greatest variation above this minimum was 31.3 per cent, his lowest 3.5 per cent, and his average 13.9 per cent.

We have no explanation to offer for the wide variation, with the possible exception of the subject showing the widest range—

<sup>2</sup> If the 1 exceptionally high day for Subject 3 is omitted (discussed below in connection with pulse rate), her average becomes 1,189 calories per 24 hours, her variation of the maximum above the minimum 125 calories, or 11.1 per cent, and her greatest variation from her average—64 calories, or 5.4 per cent.

Subject 5 with 28.8 per cent. Her average metabolism in winter (February and March) was 1,302, distinctly higher than in the summer (July) 1,145. Yet Subject 10, the only other observed both winter (early March) and summer (June), averaged the same at the two seasons (1,486 and 1,487). Another possible though not completely satisfactory reason for the differences in Subject 5 was that she was observed very early in the morning, 6.30 or 7.00, at the time when her metabolism was running lowest, and sometimes later in the day, about 11 a. m., when it ran high. She had been fasting, of course, since the night before, and had the usual rest period, but at the later date she had been attending classes and doing laboratory work before coming for the measurement of her metabolism. The late figures, however, are by no means always higher. With only a very few exceptions, all observations on other subjects were made early in the morning, usually from 7.00 to 8.30.

The diet of the subjects was not controlled, but it is not thought that there were any marked changes in it during the experiments. Neither were there marked changes of occupation nor of general health.

We wish to call special attention to the series of observations on Subject 1 (see Table VI), a young Chinese woman of excellent mentality and apparently good general health in spite of a tendency to amenorrhea. Her basal metabolism was observed daily for 26 almost consecutive days, without menstrual period. As far as we know, this is the longest series made on a woman, with the exception of Zuntz's observations. She showed much day by day variation with sudden rises and falls. There are only three subjects of the total seventeen who show a wider range than hers.

The wide range is also shown in two other women for whom we have a fairly long series of consecutive observations, Nos. 3 and 8. For No. 8 with 10 consecutive days, the variation is 11.6 per cent, and for No. 3 with 7 consecutive days, 11.1 per cent, or 19.6 per cent if the high observation of the previous month is included.

#### *Comparison with Normal Standards for Metabolism.*

The two commonly used series of "normal" standards for the basal metabolism of women are Benedict's and Du Bois'. Bene-

dict's standards, published as "multiple prediction tables"(9) are based on observations of 103 women made in the Nutrition Laboratory. They give the basal metabolism in calories per 24 hours for different ages, heights, and weights. Du Bois' (10) standards are expressed in calories per square meter per hour, and are computed from those for men on the assumption that

TABLE IV.

*Comparison of Observed Metabolism with that Predicted by Benedict and by Du Bois.*

Subject.	Heat production per day.				Heat production per sq. m. per hr.*			
	All observations.	Benedict's prediction.	Actual less calculated.	Difference.	All observations.	Du Bois' prediction.	Actual less calculated.	Difference.
	calories	calories	calories	per cent	calories	calories	calories	per cent
1	1,239	1,412	-173	-12.2	31.9	37.0	-5.1	-13.8
2	1,413	1,466	-53	-3.6	36.2	37.0	-0.8	-2.2
3	1,205	1,305	-100	-7.6	34.2	37.0	-2.8	-7.6
4	1,321	1,459	-138	-9.5	32.3	37.0	-4.7	-12.7
5	1,254	1,311	-57	-4.3	34.7	37.0	-2.3	-6.2
6	1,140	1,309	-169	-12.9	33.1	37.0	-3.9	-10.5
7	1,335	1,401	-66	-4.7	34.2	37.0	-2.8	-7.6
8	1,396	1,392	4	0.2	35.7	37.0	-1.3	-3.5
9	1,256	1,287	-31	-2.3	36.6	37.0	-0.4	-1.1
10	1,493	1,416	77	5.5	36.4	36.5	-0.1	-0.3
11	1,105	1,196	-91	-7.7	34.3	36.5	-2.2	-6.0
12	1,346	1,405	-59	-4.3	33.1	36.5	-3.4	-9.3
13	1,580	1,576	4	0.1	34.1	36.5	-2.4	-6.6
14	1,457	1,513	-56	-3.8	33.7	36.0	-2.3	-6.6
15	1,457	1,417	40	4.2	34.9	36.0	-1.1	-3.0
16	1,331	1,423	-92	-6.7	32.5	36.0	-3.5	-9.7
17	1,218	1,218	0	0.0	34.2	36.0	-1.8	-5.0
Average...				-4.1				-6.5

\* Surface area by the Du Bois height-weight chart.

the basal metabolism of women is 7 per cent lower than that of men. As the height and weight variables are included in the surface area these standards of Du Bois vary only with age, ranging for our group from 37.0 calories for 20 to 30 years to 36.0 calories for 40 to 50 years.

In Table IV the averages of all observations on our subjects are compared with these two standards. Two of our subjects

average higher than Benedict's standards, three almost exactly the same, and twelve lower. The general average is distinctly lower—4.1 per cent. In comparison with the Du Bois standard all our subjects without exception are low, ranging from -0.3 per cent to -13.8 per cent. Two are more than 10 per cent below the Benedict standard and three more than 10 per cent below the Du Bois.

We have no explanation of the low metabolism of our women. Most of them were leading fairly active lives, doing laboratory work, and some of them a limited amount of housework. They were probably not quite so active as the group of nurses studied by Harris and Benedict (9) and Palmer, Means, and Gamble (11) who also showed a metabolism below the standard. It is interesting, though of course not conclusive, that a number of people who were asked which of our group they considered most muscular all mentioned first the two individuals whose metabolism is above Benedict's standard.

#### *Pulse Rate.*

In connection with the metabolism determinations, data were collected on the pulse rate of the seventeen subjects, a total of 186 observations. They are included in Table VI and summarized in Table V. The counts were made during the experimental metabolism period after the subject had been lying down 35 minutes or more and may therefore be taken as minimum values. The averages for the different women range from 60 to 79. The average of these individual averages is 68.9, which is in rather surprisingly close agreement with that for Benedict's (9) 90 women—68.67, thus confirming his conclusion that pulse rate is higher in women than in men (Benedict's average 61.26) although basal metabolism is lower. The range of our single observations is from 54 to 100, or omitting this one extremely high case, from 54 to 88. Benedict's range is from 51 to 92.

There is no relation between pulse rate and basal metabolism for our different women (a lack of correlation also observed by Benedict), and, more surprising, there is no relation between the daily variation in basal metabolism and pulse rate for the single individual (a confirmation of Zuntz's (4) observations). The higher

pulse rates do not occur on the same days as the higher basal metabolism nor the lower pulse rates as the lower metabolism. The correlation between pulse rate and metabolism which is often spoken of is their similar increase above minimum values brought about, for instance, in exercise (12), rather than the day by day variation in minimum. We have one exception to this statement of lack of agreement—the case of Subject 3, who in one day showed the exceptional pulse rate of 100, which

TABLE V.

Subject.	Pulse.		
	No. of observations.	Range.	Average.
1	25	54- 68	62
2	12	64- 78	68
3	7	64-100	74
4	5	72- 84	79
5	22	64- 84	71
6	10	56- 78	66
7	13	68- 88	79
8	10	56- 72	65
9	11	68- 80	75
10	12	64- 78	72
11	8	62- 66	65
12	5	60- 68	65
13	10	56- 80	67
14	9	60- 68	65
15	1	60	60
16	7	68- 78	72
17	19	64- 72	67
	Total...186		Average...68.9

is 30 beats above her next count, and also her highest basal metabolism, which was 11 per cent above her average and 19.6 per cent above her minimum. This 1 day thus shows an association of tachycardia and increased metabolism similar to that recently observed by Sturgis and Tompkins (13) in hyperthyroidism. It must be remarked, however, that even this maximum metabolism of our subject is only 3.1 per cent above Benedict's prediction for her. It is to be regretted that the body tempera-

TABLE VI.  
*Fundamental Data.*

Subject.	Date.	Phase.	Pulse rate.	Oxygen per min.	Heat production.		
					Per day.	Per kilo per hr.	Per sq. m. per hr.
	1920			cc.	calories	calories	calories
1	Jan. 27	Intermenstrual.	54	176	1,220	0.871	31.4
21 yrs.	Feb. 3	"	60	184	1,280	0.909	32.8
160 cm.	" 4	"	62	183	1,270	0.906	32.7
58.5 kg.	" 5	"	62	189	1,315	0.945	33.8
1.62 sq. m.	" 6	"	60	185	1,285	0.915	33.0
	" 7	"	58	179	1,240	0.902	32.0
	" 8	"	58	173	1,200	0.856	30.9
	" 9	"	60	184	1,280	0.909	32.8
	" 10	"	60	177	1,230	0.877	31.6
	" 11	"	62	183	1,270	0.906	32.7
	" 12	"	70	173	1,200	0.856	30.9
	" 13	"	60	178	1,235	0.880	31.8
	" 16	"	62	162	1,125	0.802	28.9
	" 17	"	66	178	1,235	0.880	31.8
	" 18	"	58	175	1,215	0.865	31.2
	" 19	"	66	177	1,230	0.877	31.6
	" 21	"	64	173	1,200	0.856	30.9
	" 22	"	58	166	1,155	0.822	29.7
	" 25	"	66	170	1,180	0.840	30.3
	" 26	"	68	176	1,220	0.871	31.4
	" 27	"	68	178	1,235	0.880	31.8
	" 28	"	188	1,310	0.932	33.7	
	Mar. 1	"	60	173	1,200	0.856	30.9
	" 2	"	68	190	1,320	0.940	34.0
	" 4	"	64	185	1,285	0.915	33.0
	" 5	"	68	184	1,280	0.909	32.8
Average...			62		1,239		31.9
2	July 24	Intermenstrual.	64	204	1,420	0.888	36.3
24 yrs.	" 25	"		199	1,380	0.864	35.4
152 cm.	" 27	Premenstrual.		198	1,375	0.859	35.2
66.7 kg.	" 28	"	72	200	1,390	0.867	35.5
1.63 sq. m.	" 29	"	72	201	1,395	0.872	35.7
	" 30	"	72	200	1,390	0.867	35.5
	" 31	"	68	195	1,355	0.845	34.6
	Aug. 1	Menstrual.	64	216	1,500	0.935	38.3
	" 2	"	60	196	1,360	0.850	34.8
	" 3	"	72	207	1,440	0.898	36.8

TABLE VI—Continued.

Subject.	Date.	Phase.	Pulse rate.	Oxygen per min.	Heat production.		
					Per day.	Per kilo per hr.	Per sq. m. per hr.
	1920			cc.	calories	calories	calories
	Aug. 4	Menstrual.	68	207	1,440	0.898	36.8
	" 5	"	78	207	1,440	0.898	36.8
	" 6	"	66	214	1,490	0.930	38.1
	" 24	Intermenstrual.	64	204	1,420	0.888	36.3
Average....			68		1,413		36.2
3	Apr. 18	Intermenstrual.	70	176	1,220	1.033	34.6
25 yrs.	" 25	"		174	1,210	1.020	34.3
158 cm.	June 11	"	100	194	1,345	1.134	38.1
49.4 kg.	Aug. 8	"		170	1,180	0.995	33.4
1.47 sq. m.	" 9	"	66	173	1,200	1.017	34.1
	" 10	"	66	162	1,125	0.949	31.9
	" 11	"		180	1,250	1.055	35.5
	" 12	"	64	164	1,140	0.962	32.3
	" 14	"	64	172	1,195	1.006	33.8
	" 15	"	66	170	1,180	0.995	33.4
Average....			74		1,205		34.2
4	Aug. 25	Premenstrual.	84	198	1,375	0.893	33.7
25 yrs.	" 27	Menstrual.	84	195	1,355	0.879	33.2
164 cm.	" 28	"	76	196	1,360	0.885	33.4
64.1 kg.	" 29	"	72	184	1,280	0.829	31.3
1.70 sq. m.	Sept. 2	Intermenstrual.	80	182	1,265	0.823	31.0
	" 3	"		186	1,290	0.840	31.6
Average....			79		1,321		32.3
5	Feb. 6	Intermenstrual.	72	195	1,355	1.125	37.3
26 yrs.	" 19	Premenstrual.	72	198	1,375	1.143	37.9
161 cm.	" 20	"	66	198	1,375	1.143	37.9
50.1 kg.	" 21	Menstrual.	72	187	1,300	1.081	35.9
1.51 sq. m.	" 22	"		200	1,390	1.153	38.3
	" 23	"	72	191	1,330	1.103	36.9
	" 24	"	64	177	1,230	1.023	34.0
	" 25	Postmenstrual.	70	186	1,290	1.074	35.7
	" 26	"	72	176	1,220	1.018	33.7
	" 28	Intermenstrual.	72	193	1,340	1.112	36.9
	Mar. 21	Premenstrual.	78	200	1,390	1.153	38.3
	" 23	"		197	1,370	1.139	37.7



TABLE VI—Continued.

Subject.	Date.	Phase.	Pulse rate.	Oxygen per min.	Heat production.		
					Per day.	Per kilo per hr.	Per sq. m. per hr.
	1920			cc.	calories	calories	calories
	Mar. 24	Menstrual.		178	1,235	1.028	34.1
	" 25	"	72	172	1,195	0.992	32.9
	" 26	"	72	172	1,195	0.992	32.9
	" 27	"	68	191	1,330	1.103	36.9
	Apr. 10	Intermenstrual.	72	179	1,240	1.035	34.3
	Aug. 17	"	76	181	1,260	1.045	34.7
	" 18	"	78	174	1,210	1.005	33.4
	" 20	Premenstrual.	84	168	1,170	0.970	32.2
	" 21	"	68	158	1,100	0.912	30.3
	" 22	"	68	163	1,135	0.941	31.2
	" 23	Menstrual.	64	160	1,110	0.924	30.6
	" 24	"	78	165	1,145	0.954	31.7
	" 25	"	64	155	1,080	0.894	29.7
Average....			71		1,254		34.7
6	July 18	Menstrual.	64	173	1,200	0.945	34.8
28 yrs.	" 20	"		157	1,090	0.857	31.5
148 cm.	" 21	"	60	154	1,070	0.842	31.0
53 kg.	" 22	"		162	1,125	0.886	32.6
1.44 sq. m.	Aug. 3	Intermenstrual.	72	160	1,110	0.873	32.1
	" 5	"	66	169	1,175	0.922	33.9
	" 6	"		172	1,195	0.939	34.5
	" 7	"	66	155	1,080	0.846	31.1
	" 12	Premenstrual.	78	173	1,200	0.945	34.8
	" 13	"		165	1,145	0.903	33.2
	" 14	Menstrual.	60	174	1,210	0.951	35.0
	" 15	"	56	158	1,100	0.862	31.7
	" 16	"	64	164	1,140	0.895	33.0
	" 17	"	72	162	1,125	0.886	32.6
Average....			66		1,140		33.1
7	Aug. 1	Menstrual.	68	200	1,390	0.948	35.5
28 yrs.	" 2	"	88	189	1,310	0.898	33.6
160 cm.	" 3	"	78	189	1,310	0.898	33.6
61.0 kg.	" 15	Intermenstrual.	80	200	1,390	0.948	35.5
1.63 sq. m.	" 16	"	72	191	1,330	0.906	33.8
	" 17	"	80	193	1,340	0.915	34.2
	" 26	Premenstrual.	84	199	1,380	0.944	35.3

TABLE VI—Continued.

Subject.	Date.	Phase.	Pulse rate.	Oxygen per min.	Heat production.		
					Per day.	Per kilo per hr.	Per sq. m per hr.
	1929			cc.	calories	calories	calories
	Aug. 27	Premenstrual.	80	196	1,360	0.930	34.8
	" 28	"	84	190	1,320	0.903	33.7
	" 29	"	80	190	1,320	0.903	33.7
	" 30	Menstrual.	76	187	1,300	0.889	33.2
	" 31	"	80	186	1,290	0.883	33.0
	Sept. 1	"	76	189	1,310	0.898	33.6
Average....			79		1,335		34.2
8	May 24	Intermenstrual.	68	196	1,360	0.951	34.8
28 yrs.	" 25	"	64	212	1,470	1.033	37.8
161 cm.	" 26	"	68	196	1,360	0.951	34.8
59.6 kg.	" 27	"	56	209	1,455	1.017	37.2
1.63 sq. m.	" 28	"	68	197	1,370	0.956	35.0
	" 29	"	56	195	1,355	0.945	34.6
	" 30	"	65	215	1,495	1.043	38.2
	" 31	"	72	197	1,370	0.956	35.0
	June 1	"	68	200	1,390	0.971	35.5
	" 2	"	66	193	1,340	0.936	34.2
Average....			65		1,396		35.7
9	July 28	Premenstrual.	78	180	1,250	1.021	36.5
29 yrs.	" 29	Menstrual.	68	188	1,310	1.069	38.1
149 cm.	" 30	"	76	181	1,260	1.028	36.6
51 kg.	" 31	"	68	183	1,270	1.040	37.0
1.43 sq. m.	Aug. 25	Premenstrual.	80	177	1,230	1.005	35.9
	" 26	Menstrual.	76	182	1,265	1.033	36.8
	" 27	"	72	186	1,290	1.055	37.6
	" 28	"	80	178	1,235	1.009	36.0
	Sept. 8	Intermenstrual.	80	177	1,230	1.005	35.9
	" 9	"	78	182	1,265	1.033	36.8
	" 10	"	72	176	1,220	1.003	35.8
Average....			75		1,256		36.6
10	Mar. 11	Premenstrual.	78	222	1,540	1.043	37.9
30 yrs.	" 12	"	72	218	1,515	1.027	37.1
170 cm.	" 13	Menstrual.	72	214	1,490	1.007	36.5
61.7 kg.	" 14	"	68	205	1,425	0.962	34.9
1.70 sq. m.	" 15	"	68	211	1,465	0.990	36.0

TABLE VI—Continued.

Subject.	Date.	Phase.	Pulse rate.	Oxygen per min.	Heat production.		
					Per day.	Per kilo per hr.	Per sq. m. per hr.
	<i>1920</i>			<i>cc.</i>	<i>calories</i>	<i>calories</i>	<i>calories</i>
	Mar. 16	Menstrual.	68	213	1,475	0.998	36.2
	June 25	Premenstrual.	76	228	1,585	1.068	38.8
	" 26	"	72	205	1,425	0.962	34.9
	" 27	Menstrual.	64	231	1,600	1.082	39.3
	" 29	"	72	204	1,420	0.958	34.8
	" 30	"	76	202	1,400	0.946	34.4
	Sept. 2	Intermenstrual.		225	1,560	1.055	38.3
	" 3	"	78	218	1,515	1.022	37.1
Average....			72		1,493		36.4
11	July 25	Intermenstrual.	64	165	1,145	1.112	35.7
32 yrs.	" 26	"	64	157	1,090	1.057	33.9
151 cm.	Aug. 4	Premenstrual.	66	162	1,125	1.091	35.0
43 kg.	" 5	"	66	159	1,105	1.072	34.4
1.34 sq. m.	" 6	Menstrual.	66	156	1,085	1.050	33.7
	" 7	"	62	147	1,020	0.988	31.7
	" 8	"	66	159	1,105	1.072	34.4
	" 23	Intermenstrual.	62	168	1,170	1.130	36.2
Average....			65		1,105		34.3
12	July 11	Menstrual.	66	190	1,320	0.887	32.6
33 yrs.	" 12	"	64	188	1,310	0.879	32.2
168 cm.	" 13	"	66	199	1,380	0.930	34.1
62 kg.	" 24	Intermenstrual.	60	206	1,430	0.962	35.3
1.69 sq. m.	" 25	"	68	186	1,290	0.868	31.9
Average....			65		1,346		33.1
13	Apr. 14	Intermenstrual.		239	1,660	0.875	35.8
33 yrs.	" 15	"		229	1,590	0.839	34.3
173 cm.	" 16	"	72	237	1,650	0.869	35.5
79 kg.	" 27	Premenstrual.	80	237	1,650	0.869	35.5
1.93 sq. m.	" 28	Menstrual.	72	233	1,620	0.855	35.0
	" 29	"	72	219	1,520	0.802	32.8
	" 30	"	72	233	1,620	0.854	35.0
	May 1	"	64	218	1,515	0.800	32.7
	" 25	Premenstrual.		228	1,585	0.834	34.2
	" 26	Menstrual.	56	227	1,575	0.832	34.1

TABLE VI—Continued.

Subject.	Date.	Phase.	Pulse rate.	Oxygen per min.	Heat production.		
					Per day.	Per kilo per hr.	Per sq. m. per hr.
	1920			cc.	calories	calories	calories
	May 27	Menstrual.	64	221	1,535	0.810	33.2
	" 28	"	64	221	1,535	0.810	33.2
	" 29	"	56	214	1,490	0.785	32.1
Average....			67		1,580		34.1
14	July 22	Premenstrual.	60	200	1,390	0.766	32.1
37 yrs.	" 23	Menstrual.	64	209	1,455	0.804	33.7
162 cm.	" 24	"	68	200	1,390	0.766	32.1
75.5 kg.	" 25	"	64	209	1,455	0.804	33.7
1.80 sq. m.	Aug. 10	Intermenstrual.	66	214	1,490	0.823	34.4
	" 11	"	64	217	1,510	0.832	34.9
	" 19	Premenstrual.	66	210	1,460	0.806	33.8
	" 20	Menstrual.	64	213	1,480	0.817	34.2
	" 21	"	66	214	1,490	0.823	34.4
Average....			65		1,457		33.7
	1919						
15	Dec. 4	Intermenstrual.	60	216	1,500	0.936	35.8
40 yrs.	" 5	"		218	1,520	0.948	36.3
166 cm.	" 12	Premenstrual.		203	1,410	0.884	33.8
66.6 kg.	" 14	Menstrual.		205	1,425	0.891	34.1
1.74 sq. m.	" 15	"		210	1,460	0.912	34.9
	" 16	"		205	1,425	0.891	34.1
Average....			60		1,457		34.9
	1920						
16	Aug. 19	Intermenstrual.	72	196	1,360	0.811	33.2
41 yrs.	" 20	"	68	192	1,335	0.794	32.5
158 cm.	" 21	"	78	190	1,320	0.789	32.3
70 kg.	" 22	Premenstrual.		186	1,290	0.769	31.4
1.71 sq. m.	" 23	"	72	196	1,360	0.811	33.2
	" 24	Menstrual.		197	1,370	0.814	33.4
	" 25	"	72	180	1,250	0.745	30.5
	" 26	"	72	190	1,320	0.789	32.3
	" 27	"	72	198	1,375	0.819	33.5
Average....			72		1,331		32.5

TABLE VI—*Concluded.*

Subject.	Date.	Phase.	Pulse rate.	Oxygen per min.	Heat production.		
					Per day.	Per kilo per hr.	Per sq. m. per hr.
	<i>1919</i>			<i>cc.</i>	<i>calories</i>	<i>calories</i>	<i>calories</i>
17	Dec. 3	Premenstrual.	68	182	1,265	1.072	35.4
44 yrs.	" 4	"	72	172	1,195	1.011	33.4
162 cm.	" 5	"	64	183	1,270	1.080	35.6
49.1 kg.	" 6	"	68	189	1,315	1.113	36.7
1.49 sq. m.	" 8	"	72	165	1,145	0.974	32.1
	" 9	Menstrual.	70	189	1,315	1.113	36.7
	" 11	"		183	1,270	1.080	35.6
	" 12	"		187	1,300	1.103	36.4
	" 13	Postmenstrual.		181	1,260	1.068	35.2
	" 17	Intermenstrual.	72	185	1,285	1.090	35.9
	" 19	"	68	167	1,160	0.985	32.4
	<i>1920</i>						
	Mar. 7	"	66	170	1,180	1.011	33.3
	" 15	Premenstrual.	64	155	1,080	0.922	30.3
48.7 kg.	" 16	Menstrual.	64	164	1,140	0.975	32.1
1.48 sq. m.	" 17	"		164	1,140	0.975	32.1
	" 19	"	68	176	1,220	1.045	34.4
	" 20	"	72	178	1,235	1.057	34.8
49.5 kg.	Apr. 7	Premenstrual.	70	176	1,220	1.028	33.9
1.50 sq. m.	" 8	"		180	1,250	1.061	34.7
	" 9	"		181	1,260	1.058	34.9
	" 10	"	60	171	1,190	1.000	33.0
	" 11	Menstrual.	64	178	1,235	1.041	34.4
	" 12	"		176	1,220	1.028	33.9
	" 13	"		176	1,220	1.028	33.9
	" 14	"		169	1,175	0.987	32.6
	" 27	Intermenstrual.	72	171	1,190	1.000	33.0
	" 29	"	64	169	1,175	0.987	32.6
	" 30	"	64	181	1,260	1.058	34.9
	May 1	"		169	1,175	0.987	32.6
Average....			67		1,218		34.2

ture was not determined on this day. None of our other pulse rates is within the range of pathological significance.

The variation of pulse rate during menstruation is almost as irregular as the basal metabolism. In three cases Subjects 2, 7 (first period observed), and 10 (first period observed), there is

a slightly higher pulse rate the first of the menstrual period than later, an observation in agreement with King (14), who found that pulse rate and temperature follow the rhythmical movement in life processes, while blood pressure, systolic and diastolic, and pulse pressure varied irregularly. In most of our subjects, however, no regular variation was observed.

#### SUMMARY.

1. Series of 216 observations are given on the basal metabolism of 17 women, 14 of them including 1 or more menstrual cycles, and 1 being observed for 26 almost consecutive days.

2. There is no definite change in the basal metabolism during menstruation. This is seen from the facts that the average of the intermenstrual and menstrual observations is almost the same, and that no rhythmical periodic variation in metabolism can be noted.

3. The daily variation for each subject is great, ranging from 7.4 to 28.8 per cent, or an average of 13.2 per cent. This is slightly less than the average which Benedict found, 13.9 per cent, with a group of individuals observed 5 days or more. Erroneous conclusions can easily be drawn from metabolism observations unless measurements are made on more than 1 day.

4. Most of our subjects show a somewhat lower basal metabolism than that calculated for them from the Benedict or the Du Bois standards.

5. There is no relation between minimum pulse rate and basal metabolism in our subjects, except in one case for 1 day where the pulse rate increased to an extent which may be considered pathological. Neither is there definite constant change in the pulse rate during menstruation.

The authors wish to express their thanks to Dr. F. G. Benedict for reading and criticizing the manuscript of this paper, to Dr. T. M. Carpenter for helpful suggestions at the beginning of the work, and to the women who served as subjects, through whose interest and cooperation the investigation was made possible.

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## FAT-SOLUBLE VITAMINE.

### VII. THE FAT-SOLUBLE VITAMINE AND YELLOW PIGMENTATION IN ANIMAL FATS WITH SOME OBSERVATIONS ON ITS STABILITY TO SAPONIFICATION.\*

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(Received for publication, April 12, 1921.)

When in the early part of 1919, we (1, 2) formulated the working hypotheses, in our fat-soluble vitamine investigation, that the vitamine might be identical with, or closely related to certain yellow pigments of the carotinoid type, we made the attempt to correlate its occurrence on this basis, to formulate a procedure for its isolation, and to collect information as to its possible chemical nature. In these objects we have been substantially aided by our hypotheses, for while all the possibilities anticipated have not materialized yet there has been given a direction to our experimental efforts in this field, and apparently to those of others, which has lead to great centralization of effort. It was obvious that, in spite of the numerous instances of association of the physiological growth-promoting property which is attributed to the presence of fat-soluble vitamine and yellow plant pigments, the two would not necessarily have to be identical, in fact there need be no material relationship in composition or structure, as their coincident occurrence in nature might be due to physiological determination, pure and simple. In this event, with the diversification of metabolic processes which obtain in the plant and animal kingdom, it was to be expected that sooner or later the fat-soluble vitamine would be found to be present in a menstruum entirely free from pigments of the carotinoid type. To run across such an instance, appears to

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have been the good fortune of Palmer and Kempster (3), who demonstrated that pork liver, rich in the vitamine, contained no pigments of the aforementioned character. Gross of this laboratory has obtained similar results<sup>1</sup> but Rosenheim and Drummond (4) purport to have shown that while carotin and xanthophyll are absent, another pigment of this type is present. It may still be said that in such instances where the fat-soluble vitamine is found to occur in the absence of color the pigment is present in the leuco form (2). While this can be accepted as a possibility it is at present not worthy of serious consideration because nothing is known of the structure of the carotinoid molecules and therefore nothing is known as to the probability of the existence of leuco compounds in the series. Nevertheless, as far as studies in this domain have been pursued, both in regard to distribution of vitamine and pigment and in regard to their physical and chemical properties, there is left no doubt but that chemically and physiologically they are related. These relations will be discussed in succeeding papers in which our results will be correlated with those of others who have been active in this field of investigation. In this paper it is desired to present data on the fat-soluble vitamine content of various animal fats as correlated with yellow pigmentation, and to record a few observations made on its stability to saponification.

#### EXPERIMENTAL.

For the laboratory technique employed in these experiments the reader is referred to previous papers of this series (5-10), as the experimental methods employed were essentially the same. In no instance has it been deemed permissible to make any selection of data. Four animals, usually two males and two females, were started in each group and continued until death or until the differences among the groups became so pronounced as to make further continuation superfluous. In a few instances where data were purely confirmatory we have felt at liberty to present merely representative illustrations.

Failure of growth alone has not been accepted by us as a good criterion to use in establishing the lack of the fat-soluble vitamine

<sup>1</sup> Unpublished data.

and this for very obvious reasons. Emphasis was, therefore, always placed upon the appearance of the eyes of the rats, as an inflamed condition, an ophthalmia variously termed a conjunctivitis, a xerophthalmia, and a keratomalacia, makes its appearance in the vast majority of animals on a fat-soluble vitamin-poor diet as first pointed out by Osborne and Mendel (11). Though the exact condition which is incident when the eyes become infected is not described accurately by these terms we have used them rather indiscriminately in the past hoping that pathologists would soon describe the condition in detail and designate it accurately.<sup>2</sup> Stephenson and Clark (12) introduced the term keratomalacia in preference to xerophthalmia, apparently because in these inflammatory reactions a softening of the cornea with loss of the lens frequently results. In a certain sense each of these terms has its proper application in the various types or stages of the ophthalmitis observed. Generally a conjunctivitis with an erythema and edema of the eyelids appears first, later a keratitis or inflammation of the cornea results, and ultimately, if the diet is not corrected or if resistance to the infection does not develop of its own accord especially in severely purulent inflammatory reactions, a dryness or xerosis of the eye—a xerophthalmia—marks the culmination of the eye symptoms. As usually observed and especially in the primary stages the condition is marked by an excessive secretion and is certainly not a xerophthalmia. We believe that, in most instances when the inflammation is not confined to the conjunctiva, the infection is most accurately designated as a keratoconjunctivitis. Only occasionally will we have reason to speak of a xerophthalmia because true xerophthalmia has rarely made its appearance in our colony. When the inflammatory reaction is so severe as to cause permanent injury and the diet is not corrected, death results rapidly; if it is transitory, making its appearance now and then—indicative of only a partial deficiency of the fat-soluble vitamin—then practically complete recovery has always been observed. The recovery cannot be considered entirely complete because an inflamed condition of

<sup>2</sup> Such studies have recently been published by Wason (Wason, I. M., *J. Am. Med. Assn.*, 1921, lxxvi, 908), since the preparation of this manuscript.

the eyes, beyond a mere infiltration of fluid—an edema of the conjunctiva and eyelids—leaves its permanent mark in the destruction of the hair follicles on the lids. This “bare eyed” condition always arouses our suspicions of a fat-soluble vitamine deficiency, suggesting that a transitory reaction has escaped our notice if the inflammation was not actually observed.

In addition to the inflammation of the eyes, we have observed in many animals, large and small, an apparent resistance to infection of the eyes even though continually exposed by contact to severely infected animals, all on a fat-soluble vitamine-free ration. In such individuals an enophthalmia or a “small eyed condition” is frequently observed. The eyeballs are not “beaded” as in normal rats but appear small and sunken in the orbital cavity. Many of these individuals succumb to respiratory infections, in fact we have been led to think that possibly a certain immunity to infection of the eyes is thus conferred.

The incidence of respiratory infections as part of the syndrome induced by fat-soluble vitamine deficiency was described by McCollum (13) in his early work. It may consist, as we have observed, of a nasal or bronchial catarrh or even pulmonary infection with mucous or purulent exudate, at times even resulting in hemorrhage. Animals thus afflicted in the early stages of the disease sneeze and cough violently but later as the inflammation becomes confined more to the lungs the cough subsides and dyspnea becomes very pronounced with the slightest activity. Such animals fail very rapidly and even with the introduction of fat-soluble vitamine in the ration rarely show normal growth subsequently.

A fat-soluble vitamine deficiency is also far from being conducive to normal cutaneous nutrition so that very often—especially after an age of 4 months has been reached—evidence of dermal malnutrition makes its appearance. The fur appears bushy and thin, cutaneous growths occur on the tail, ears, and nose, and finally sores, which heal with difficulty, appear on the feet, limbs, and body; all bear testimony to this state of malnutrition.

All of the aforementioned symptoms and conditions have been carefully watched and noted at the time of the weekly weighings of our animals unless their condition or the nature of the

experiment made examinations at shorter time intervals of moment. Judgment was based on the sum total of indications.

*Fat-Soluble Vitamine Content of Cod Liver Oil.*

Since Osborne and Mendel (14) in their pioneering studies on the fat-soluble vitamine found cod liver oil to contain this dietary essential surprisingly little experimental endeavor has been made to study it in its quantitative relations though repeated emphasis has been placed on the efficacy with which it can be used as a therapeutic agent in perverted metabolism of the osteoid tissues. As far as known to the writers, Zilva and Miura (15) are the only investigators who have studied this problem. They have recently published a preliminary note on their experiments stating that cod liver oil in its crude state was found to be 250 times as potent as butter in furnishing this constituent.

Our investigations were not outlined to bring out such extreme differences as we were primarily intent on a correlation of the fat-soluble vitamine with pigment content in comparison with the intense pigmentation of butter, and selected cod liver oil because we had at hand an excellent sample which in comparison with butter was practically devoid of yellow pigments—it had only a faint yellowish green color. If the vitamine were a yellow pigment it should therefore have shown only limited activity. The sample of oil was prepared from fresh cod livers with minimum heat exposure. The livers were cut into small pieces, put into a steam-jacketed open cooker, and heated not to exceed 180° F. The oil collecting on the surface was skimmed off as rapidly as it formed in the first 35 minutes of heating, filtered through paper, and bottled.

As seen in Chart 1, this oil when fed at a level corresponding to 2 and 5 gm. of material in a kilo of ration was exceedingly active; it was far more efficient than any sample of butter fat that we have ever studied. This alone was one of the things which made us very skeptical in the early course of our studies of the assumption that the fat-soluble vitamine was necessarily a yellow pigment.

*Seasonal Variation in the Fat-Soluble Vitamine Content of Butter.*

Much has been written and said about the fat-soluble vitamine content of butter fat primarily because of its extensive use as an article of food and therefore its probable importance as a source of the fat-soluble vitamine in the human dietary. Due to the comparatively low intake of butter fat in the diet of the adult, the destruction of its vitamine in some cooking processes, the general occurrence of the vitamine in many foods such as green plant tissue, certain seeds and even roots, and the undetermined requirements of man, it is questionable whether its vitamine content would now attract the attention that it does if it were not for the fact that butter fat was the material in which the vitamine was first discovered. Early in our studies we were impressed with the fact that butters varied decidedly in their vitamine content; most of them being very rich in this dietary constituent, but some being as poor as the average oleomargarine (5). Certain observations which we made also impressed us with the lability of the vitamine as we found it destroyed in heated butters and in butters kept under poor storage conditions (5), so that we did not feel at liberty to draw conclusions with respect to the primary causal factors involved.

As our studies on the distribution of the fat-soluble vitamine progressed and indications of the occurrence of the vitamine with yellow pigments were obtained it appeared profitable to attempt to correlate these relations in butter fat especially in view of the fact, as is well known in dairy practice, that butter churned in late winter or early spring under Wisconsin conditions without artificial coloring is practically void of all color. If the vitamine content should be demonstrated to be of the same order of magnitude as the pigmentation then further presumptive evidence of this relation would have been obtained.

To this end there were prepared samples of butter fat in the latter part of the months of March, April, May, and June from cream obtained at the University creamery which was representative of the composite collection from a large number of dairy farms in the vicinity. The butter was churned in the laboratory, melted, and filtered at a low temperature, and then stored in a refrigerator till used in the experiments. The butter

fat was fed in basal rations which have been repeatedly demonstrated to be very low in the fat-soluble vitamine content, though controls were never omitted. Originally, through the March series of experiments, the basal ration was one which we have used before, consisting of: casein, 18; agar, 2; Salts 32, 4; ether-extracted wheat embryo, 6; and dextrin, 70. In the April, May, and June series a white corn ration, consisting of: white corn, 40; casein, 14; Salts 32, 3; Salts 35, 1; and dextrin, 42; was used. This latter is an excellent ration and guarantees a sufficiency of the water-soluble vitamine being introduced with the white corn which is not always the case when a variable commercial product such as wheat embryo is used as its source. The butter fats of unknown value were introduced in these rations at the expense of so much dextrin.

As illustrated in Charts 2, 3, and 4 there occurs a decided variation in the vitamine content of the different butters; but even gross inspection of the monthly collections made it evident to us that the variations in vitamine were not quantitatively reconcilable with the variations in pigment. To enable more accurate comparisons to be made we availed ourselves of the use of a standard color solution in a Duboscq colorimeter, which was prepared by dissolving 7 gm. of  $K_2CrO_4$  and 0.074 gm. of  $K_2Cr_2O_7$  in water and making it up to 100 cc. volume. Such a solution compares favorably with the color of June butter fat but as the intensity of pigmentation is reduced, as is the result in the winter butter fat, the effect of a residual yellowish green pigmentation becomes disturbing and comparisons are not so easily made. Nevertheless, the accuracy of the determinations exceeded by far the requirements of our work as the results of the feeding trials themselves cannot be evaluated with any great degree of accuracy. With June butter fat accepted as having a value of 100, May butter fat was found to have a value of 86, and March and April butter fats, a value of 2.8. The latter were therefore practically colorless. With these factors in mind, upon inspection of the growth curves, it becomes increasingly evident that the fat-soluble vitamine content of the butter fats does not run parallel to the intensity of pigmentation; otherwise in the first place May and June butter fats fed at the 0.5 per cent levels should have been far more potent than they actually were

in comparison with the March or April butter fats as they carried from 30 to 35 times as much pigment. In the second place when fed at different levels, 2 per cent for the March and April butter fats as compared with 0.5 per cent for the May and June butter fats, the former should not have exceeded the latter in efficiency as even then only from one-eighth to one-ninth as much total pigment had been introduced into the ration. These findings harmonize with those of Drummond and Coward (16) who arrived at similar conclusions.

Before the facts of these relations were obtained many attempts were made in the summer of 1919 to ascertain if any parallelism between vitamine and pigment content obtained by taking advantage of the fact that the carotin in butter fat is easily destroyed by heating. We heated butter fat in deep and in shallow dishes in the presence and absence of oleic acid—as acids accelerate pigment destruction in butter very markedly—with and without aeration with hydrogen, carbon dioxide, and air. We expected that if vitamine and pigment were not identical under some of these conditions destruction of the one without destruction of the other might be found to occur. Our results were entirely unsatisfactory as consistent duplication of results on different samples could not be obtained. As the selection of data bearing out a particular point at issue is not justifiable when unexplainable contradictory evidence is also obtained, the results of this work were not published. They served to convince us, however, that the success of such experimental attempts depended largely upon good fortune as butter fat is too variable in fat-soluble vitamine content to be taken as a good source of vitamine for studies of this character.

Since these experiments were carried out, Stephenson (17) has submitted data which tend to show that charcoal can be successfully employed in the removal or destruction of the pigment without causing complete destruction of the vitamine. Unfortunately her experimental period is shorter than desirable, especially as she worked with animals of considerable size in which we have found under normal conditions the vitamine reserve to be high and continued normal growth for 8 weeks to be common. The sudden death of one individual is not reassuring as pulmonary infections carry off some individuals on



a fat-soluble vitamine-poor diet without premonitory symptoms in the course of a few days; nevertheless her data are very suggestive especially in view of Palmer's (3) observations on the feeding of pork liver.

*Fat-Soluble Vitamine in Beet Fats.*

Osborne and Mendel (18) and later Halliburton and Drummond (19) showed that beef fats might contain considerable amounts of the fat-soluble vitamine though in general their efficiency in furnishing this dietary constituent was not to be compared with butter fat. By fractionally crystallizing the beef fats from alcohol, Osborne and Mendel obtained a very active fraction. The beef oils were found to be exceedingly active while the solid residue was inactive. In our work a somewhat similar product, the oleo oils from beef fats, prepared in commerce for the manufacture of oleomargarine, were in some instances found richer in the fat-soluble vitamine than many butters (5). Subsequent to the publication of these results we became aware of the fact that the vitamine content as determined in our feeding experiments with these samples seemed to vary directly with the intensity of pigmentation. This led to the collection of additional data to determine if this was a mere coincidence or if it was commonly true.

During 1919 the experiments were confined to the investigation of the perinephric fat of animals of the Jersey, Durham, and Holstein breeds. The fatty tissue was ground in a meat hasher and extracted by heating slightly above the melting point in a steam oven and then straining and decanting the melted fats. They were preserved in Mason jars in a refrigerator until utilized in the experiments.

As seen in Chart 5, the Jersey fat was very active while the Durham fat gave no evidence of containing this vitamine. The same inactivity was shown by the Holstein fat. Both the Holstein and Durham fats were practically colorless; the Jersey fat, on the other hand was fully as pigmented as a sample of June butter.

In 1920 we duplicated these experiments except for the fact that the samples were not taken from any particular breeds

but were selected promiscuously from slaughtered animals for color intensity. The dark beef fat was fully equal in color to June butter, the medium beef fat was two-thirds as colored and the light beef fat only one-tenth as colored. These values were obtained by measurement in a Duboseq colorimeter. The results shown in Charts 6 and 7 are essentially of the same character as those obtained the year before—the fat-soluble vitamine content roughly parallels the pigmentation. In view of the results that we have obtained with butter fat, it is not to be concluded that this is necessarily always the case. The rapidity of fat deposition, its mobilization, and the variation in the assimilation of pigment with different breeds and individuals, no doubt all operate to modify the primary determinative effect of the composition of the ration. Just how the latter may influence the relations we have again had occasion to observe with the fat-soluble vitamine content of egg yolks.<sup>1</sup> Normally, on ordinary rations light-colored yolks are low in the fat-soluble vitamine; yet by the selection of a special and unusual ration we have succeeded in producing light-colored yolks of normal vitamine content.

*Stability of Fat-Soluble Vitamine to Saponification.*

The study of the characteristics of the fat-soluble vitamine has presented considerable difficulties particularly due to mistaken notions of its stability and solubility properties which were fostered by suggestions rather than conclusive evidence as presented by various investigators. McCollum and Davis (20) reported the transference of the fat-soluble vitamine from butter fat into olive oil after the butter fat had been submitted to a mild saponification at room temperature. This was submitted as a preliminary paper in 1914 as it was stated that other experiments were under way and would be reported as soon as advisable. In the experiments detailed by them a number of difficulties can be appreciated which have made duplication very difficult as no confirmation of these attempts has been published. In the first place the drying of the soaps and the dissipation of the ether vapors from the ether-olive oil extract are processes not easily carried out under laboratory conditions without causing the destruction of considerable amounts of the vit-

amine. In the second place the olive oil extract as fed at a 3 per cent level and, therefore, equivalent to 6 per cent butter fat, did not leave a sufficient margin of fat-soluble vitamine to guarantee its presence at the close of operations as many samples of butter fat without having been subjected to any treatment are ineffective when fed at this level. In the experiments mentioned, however, it is possible that the 20 per cent lactose (21) carried considerable vitamine so that but a small increment was needed to elicit a growth response. Nevertheless, all these facts made it appear very unprofitable to attempt to repeat these experiments especially as the vitamine was ultimately brought into the solution of a fat with no determination of the completeness of the saponification beyond an inspection of the solubility of the reaction mixture which in the presence of so much soap is far from satisfactory. These experiments are therefore to be considered merely as a demonstration of the resistance of the fat-soluble vitamine to the mild saponification employed. In the light of this it was not surprising that Drummond (22) failed in demonstrating the resistance of the vitamine to the mild saponification of Henriques used by McCollum. He varied his procedure in that he attempted an ether extraction of the soaps but failed to show any activity of either the extract or an ether extract of the saponified residue when he fed the equivalent of 15 and 20 per cent of butter fat and whale oil, respectively.

From our work on the extraction of the vitamine from plant materials, where we adopted the method of separation in use for the carotinoids, we have demonstrated repeatedly the resistance of the vitamine to saponification and its subsequent extractibility by ether (10). From our present work (Chart 8), it is evident that the fat-soluble vitamine as found in animal fats has similar properties. In two instances, Lots 741 and 969, the saponification was conducted at 37°C. for 4 hours; 300 gm. of the fat being treated with 600 cc. of 20 per cent alcoholic potash, which are the proportions of fat and alkali used in the methods of analysis of The Association of Official Agricultural Chemists (23). At the end of the 4 hour period 2,400 cc. of water were added and the aqueous alcoholic solution of soaps extracted three times with ether. The ether extracts were washed with a small volume of water and then evaporated

directly at room temperature in an air current on the ration. For control purposes a saponification was carried out with butter fat parallel to one run by the Official Methods using the same concentration and excess of alkali, acting for the same period of time at the same temperature as the preparation. They gave the same saponification value indicating that the saponification in our butter fat preparation was complete. Nevertheless, we made another preparation in which the fat was boiled with 20 per cent alcoholic potash for one-half hour as required by the Official Methods but with no reduction in alkali concentration. The growth curves of Lot 972 bear testimony to the fact that even under these drastic conditions the fat-soluble vitamine was not destroyed to any appreciable extent. From this it can be concluded that it is not a fat or an ester and that it is not labile to heat in the presence of a high concentration of alkali.

We desire to express our appreciation to Lord Brothers who furnished the cod liver oil and Armour and Company who furnished us with the beef fats.

#### SUMMARY.

In cod liver oil there is present a very high concentration of the fat-soluble vitamine with but small amounts of yellow pigments.

Butter fat shows a seasonal variation in the fat-soluble vitamine content when obtained from stall fed cows during the winter and pastured in the summer as is the practice under Wisconsin conditions.

The fat-soluble vitamine content of butter fat does not run closely parallel to the yellow pigment; yet in general, due to determination by their content in the feed, butters highly pigmented are rich in the vitamine; butters low in pigment should be looked upon with suspicion.

In beef fats the relations are somewhat similar; those most pigmented are also generally richest in their fat-soluble vitamine content.

The fat-soluble vitamine withstands severe methods of saponification. This indicates that it is not a fat and probably

not an ester and makes possible the compounding of satisfactory fat-free synthetic rations for investigative purposes.

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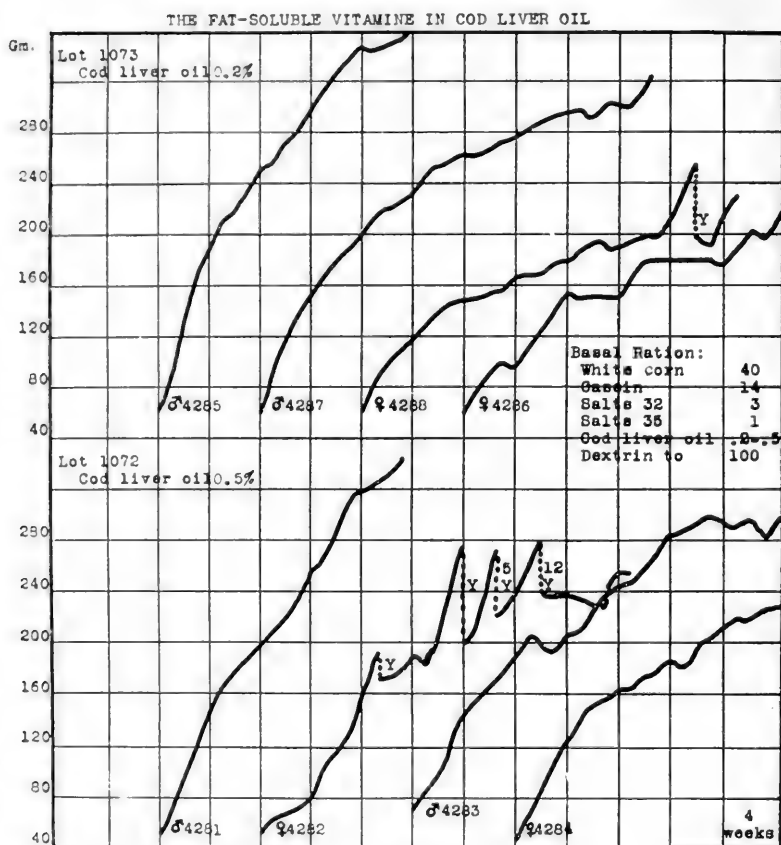


CHART 1. Lots 1072 and 1073 illustrate the remarkable growth-promoting property of small amounts of cod liver oil as a source of the fat-soluble vitamine. Rats 4285 and 4281 both continued their phenomenal rate of growth beyond the curves of growth shown here, the former weighing 395 gm. and the latter, 422 gm. 6 weeks later. Rat 4282 raised three young out of a litter of twelve in 5 weeks to an average weight of 40 gm. None of the other litters was raised. White Indian corn at a 40 per cent intake level was used as the source of water-soluble vitamine as it has been shown in numerous experiments to lead to nutritive failure as a source of the fat-soluble vitamine and yet it furnishes plenty of the water-soluble vitamine for normal growth.

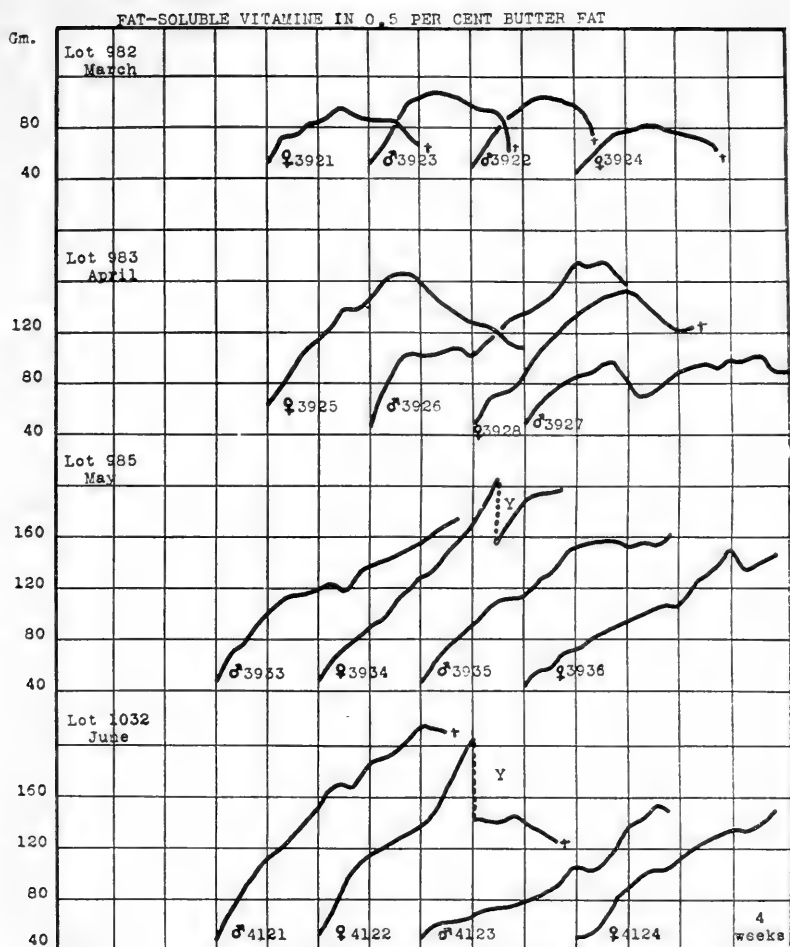


CHART 2. This chart shows the variable effects in growth responses when an attempt is made to introduce the fat-soluble vitamine into the ration by the substitution of 0.5 per cent of butter fat collected in successive months for 0.5 per cent of dextrin. On the March collection in Lot 982 by the pronounced failure of growth and even maintenance in all the animals, by the dermal infections of Rats 3921, 3922, and 3923 and by the ophthalmia in Rats 3922, 3923, and 3924, there is left no doubt that a deficiency of the fat-soluble vitamine obtained. On the April collection, Lot 983, growth was considerably better but all the animals were afflicted with keratoconjunctivitis. On the May butter fat, Lot 985, growth was continuous but dermal nutrition was poor; no eye symptoms were observed. On June butter fat, Lot 1032, the experimental results were practically the same except that dermal malnutrition was not evident, and Rat 4122 contracted a prolonged bronchial infection. Young were not reared.

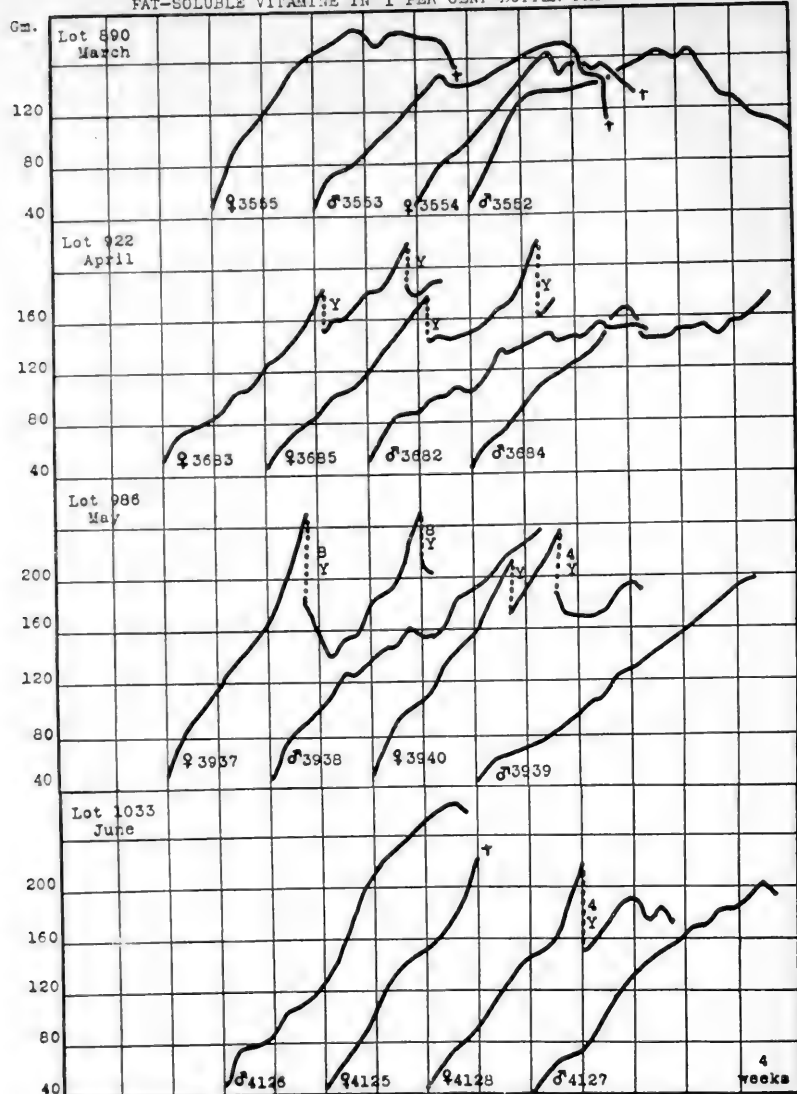


CHART 3. When the butter fat was increased to 1 per cent of the ration, growth was in all cases much improved with marked evidence of inferiority of the March product as fed in Lot 890. All of the rats contracted keratoconjunctivitis before their death and Rat 3553 by its sneezing and coughing indicated the presence of a respiratory infection. The April sample, although not up to par, was evidently richer in fat-soluble vitamine content, as the growth performance of the animals was much better and no indisputable symptoms of conjunctivitis were observed. There were, however, some indications of cutaneous malnutrition as the tails of the animals bore some infections towards the close of the experimental period. For May and June, Lots 986 and 1033, no special comments as to the normality of growth appear to be called for. On the May product Rat 3940 even raised a litter of four without apparent difficulty.



# FAT-SOLUBLE VITAMINE IN 2 PER CENT BUTTER FAT

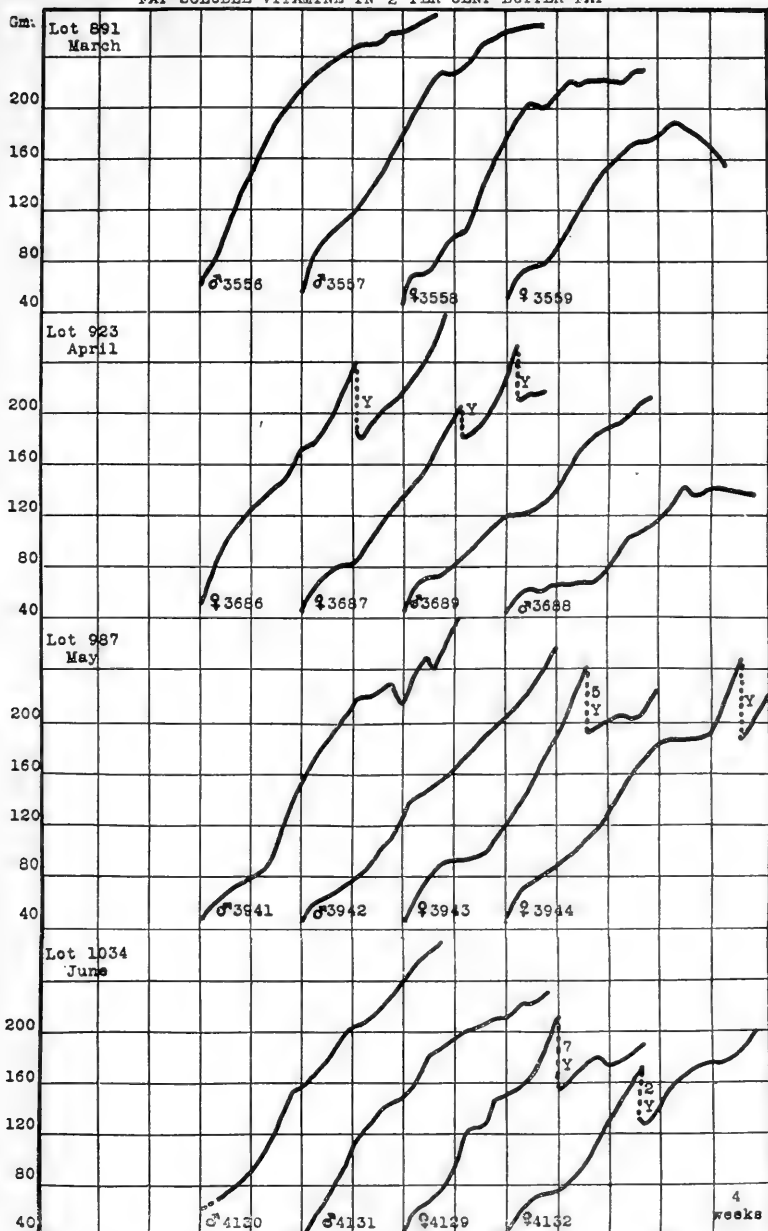


CHART 4. With the butter fat increased to 2 per cent of the ration deficiencies in the nutritive value of the collections, as far as evident were entirely eliminated, if we except the fact that Rat 3559 failed due to a localized caseous pulmonary infection. All other individuals remained normal.

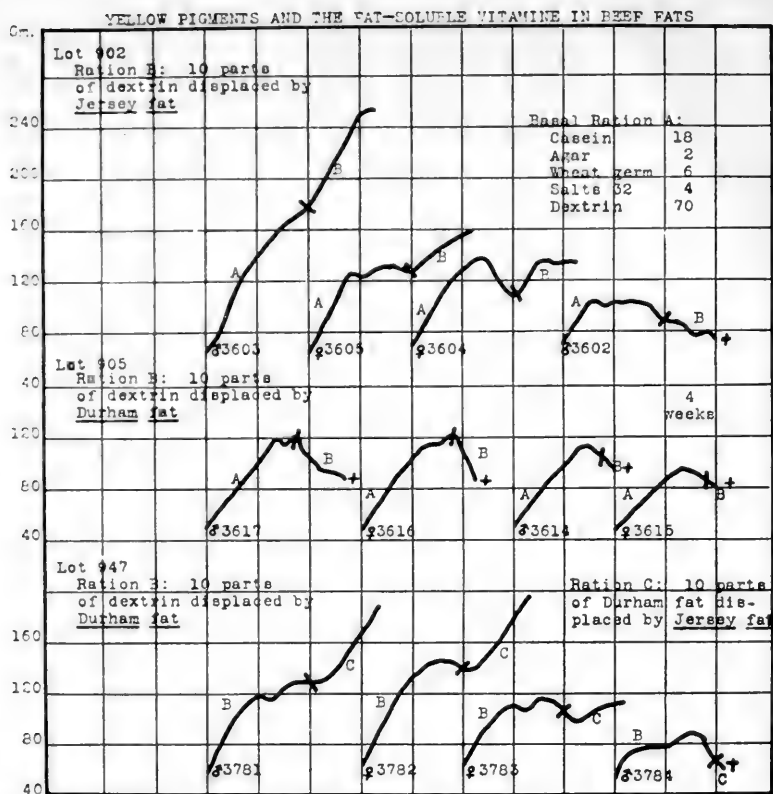


CHART 5. This chart illustrates the growth performance of rats on the perinephric fats of cattle as a source of the fat-soluble vitamin. In Lot 902, Rats 3602 and 3604 were afflicted with keratoconjunctivitis, the eyes of the others being normal when the change to Ration B carrying the Jersey fat was made. Except for Rat 3603 the condition of the animals was poor so that the response to the improvement of the diet was probably not of the order of magnitude to be expected. This is suggested by the results shown by Lot 947.

Lot 905. When the rats of this lot were changed to the fat-containing ration, all of the rats were affected with ophthalmia. The eyes of only Rat 3617 showed improvement subsequent to the change before death ultimately supervened. This was the only suggestion of the possible presence of the fat-soluble vitamin; possible because improvement of eyes is sometimes though not generally observed without any change having been made in the diet, yet growth in such cases is not restored and death slowly results.

Lot 947 when started out on a ration containing ten parts of Durham fat, such as was substituted in the ration of Lot 905, did not enable the rats to grow any longer or better than when the fat was not included. All of the rats showed inflamed eye conditions which promptly subsided shortly after the change to Jersey fat was made. This was true even in the case of Rat 3784 which however, due to its impoverished condition, died shortly thereafter.

# YELLOW PIGMENTS AND THE FAT-SOLUBLE VITAMINE IN BEEF FATS

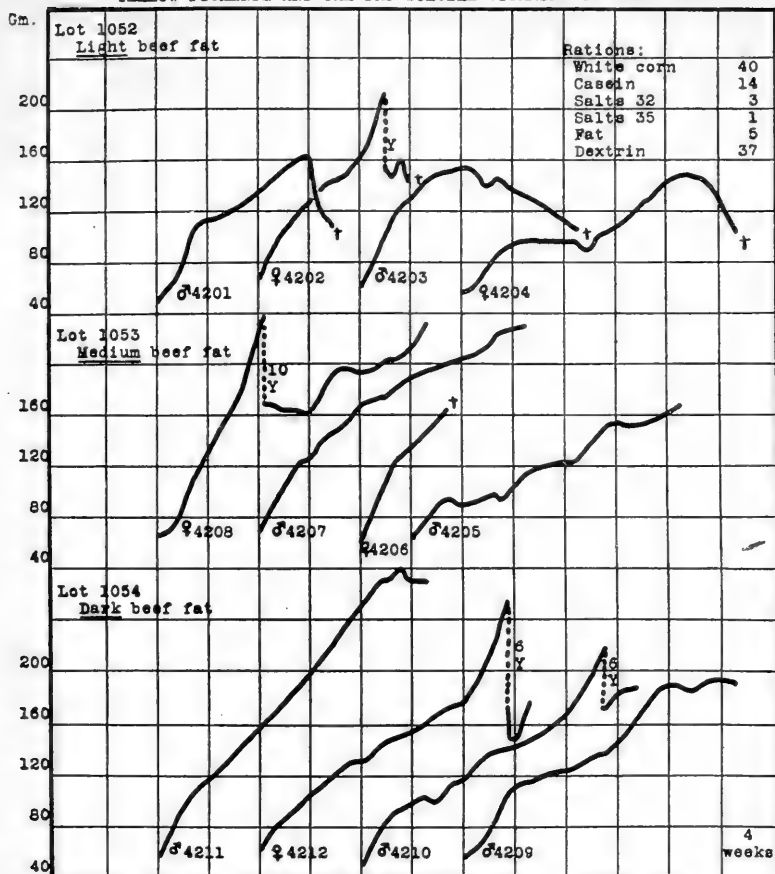


CHART 6. This chart shows the growth observed on the 1920 beef fat samples when the animals were given the fat addition from the beginning of the experiment. In Lot 1052, on the light beef fat samples, Rat 4201 developed pulmonary infections and Rats 4203 and 4204, keratoconjunctivitis. The appearance of Rat 4202 alone remained fairly normal but rapid failure ensued after parturition which is very often observed on a fat-soluble vitamine-poor ration. In Lot 1053 on the medium beef fat Rat 4205 contracted conjunctivitis after 16 weeks on the ration, later by the 20th week its eyes turned purulent with complete recovery by the 26th week, but by that time indications of dermal malnutrition were very distinct; it showed loss of hair, localized infections on body, and a horny epithelial growth on its nose. Rats 4208 and 4207 gave indications of dermal malnutrition only. These conditions in general suggest a deficiency in the fat-soluble vitamine content even though growth was fairly good. In Lot 1054 on the dark beef fat all the animals maintained themselves in good condition to the end of the experimental period.

# YELLOW PIGMENTS AND THE FAT-SOLUBLE VITAMINE IN BEEF FATS

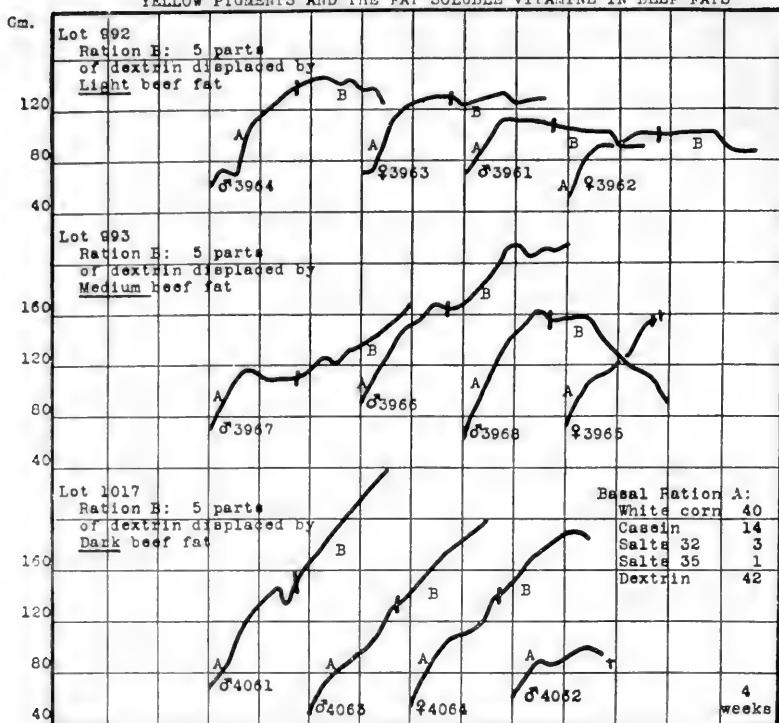


CHART 7. Chart 7 illustrates the relative efficiency of the same beef fats illustrated in Chart 6 as determined by their ability to induce recovery in rats which had indisputable evidence of a fat-soluble vitamine deficiency in the ration.

In Lot 992, Rats 3961 and 3963 had edematous eyes, respectively, at the end of the 6th and 7th weeks on the white corn ration. In the case of the former, temporary improvement was noted from time to time; in the latter, permanent improvement extended over a period of 6 weeks, but in neither case was growth resumed. This illustrates, what we have often observed, that in the vast majority of cases less of the vitamine is required to maintain normal eye conditions than to maintain growth. In Rats 3962 and 3964 the eyes were inflamed severely at the time of discontinuation of the trial.

In Lot 993, on the medium beef fat, Rat 3967 indicated incipient inflammation with slight edema of the conjunctiva which was promptly cured upon change of ration. Rats 3966 and 3968 also showed some indications of an edema; in the former it persisted in spite of change of ration.

On the dark beef fat in Lot 1017 previous to the change all rats except Rat 4061 showed a severe ophthalmitis. Rat 4064 showed an enophthalmia. In all cases where the change of ration was made improvement was prompt and recovery complete.

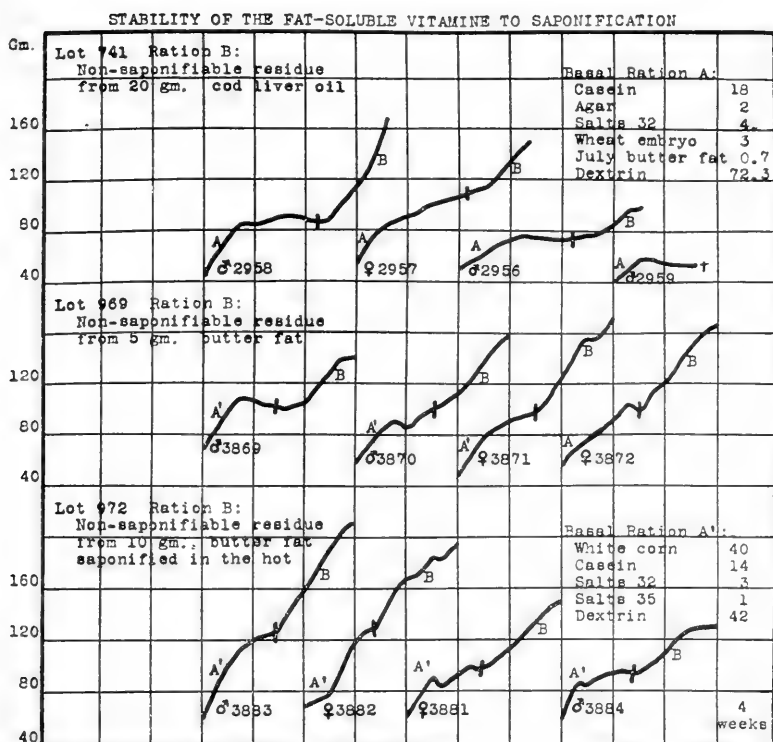
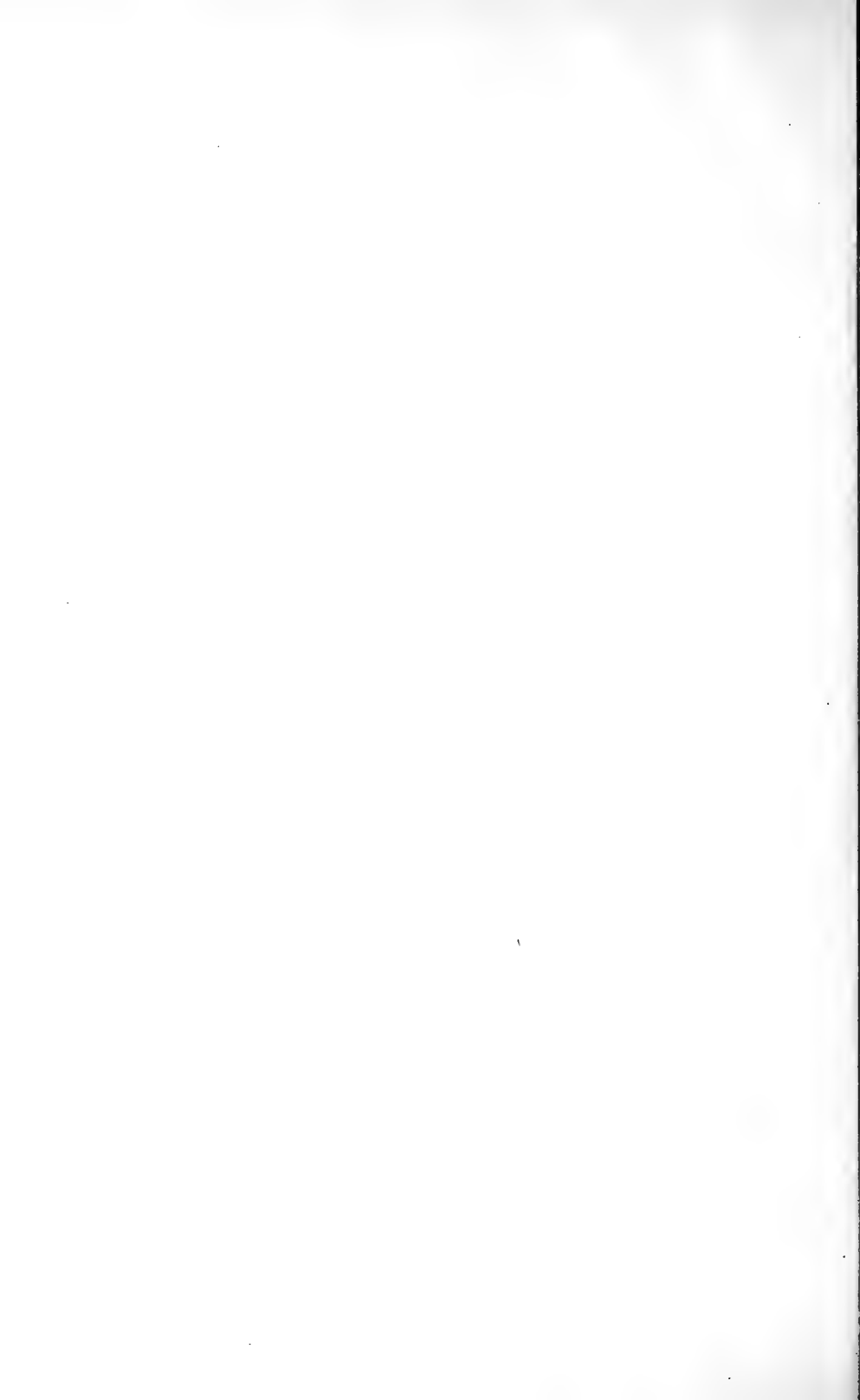


CHART 8. This chart shows the resistance of the fat-soluble vitamine to destruction by saponification of the fats in cod liver oil and butter fat as indicated by the prompt recovery of growth in the rats when an ether extract of a solution of the soaps was added to the basal rations. This consisted, in Lot 741, of a ration which had served in an experimental series to determine the fat-soluble vitamine content of butter fat. It is noteworthy that 0.7 per cent of this sample of July butter fat furnished no appreciable amounts of the vitamine. In Lots 969 and 972 our usual white corn ration was used. In no case, previous or subsequent to the change of ration, was any abnormality of eye conditions observed.



## SUPPLEMENTARY PROTEIN VALUES IN FOODS.

### I. THE NUTRITIVE PROPERTIES OF ANIMAL TISSUES.

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(Received for publication, March 21, 1921.)

In 1915 McCollum and Davis<sup>1</sup> described a systematic procedure for evaluating each of the several essential factors in foodstuffs. This procedure involves the feeding of the food under consideration as the sole source of nutriment to one group of animals, and to another the same food supplemented with single and multiple additions of purified foodstuffs in every possible combination. Such additions include protein, inorganic salts, a source of fat-soluble A, water-soluble B, and as was later pointed out by Chick and her coworkers,<sup>2</sup> Cohen and Mendel,<sup>3</sup> and others, water-soluble C. The latter factor is not essential in the diet of the rat. This procedure constitutes a biological method for the analysis of a foodstuff, and has been adopted by several students of nutrition. It has yielded results which have profoundly changed our basis of judgment as to the quality of a diet.

Studies from several laboratories have established the general landmarks which enable us to appreciate the lines of procedure which must be followed if satisfactory diets are to be made up by combining the various types of animal and vegetable foods. In order to make such combinations of foodstuffs it is necessary that we should understand in detail the special qualities of each of the important natural foods. Such an understanding can be secured only through carefully planned experiments on animals in which

<sup>1</sup> McCollum, E. V., and Davis, M., *J. Biol. Chem.*, 1915, xxiii, 181.

<sup>2</sup> Chick, H., and Hume, E. M., *Tr. Soc. Trop. Med. and Hyg.*, 1917, x, 141. Chick, H., Hume, E. M., and Skelton, R. F., *Biochem. J.*, 1918, xii, 131.

<sup>3</sup> Cohen, B., and Mendel, L. B., *J. Biol. Chem.*, 1918, xxxv, 425.

each food is studied as the sole source of nutriment, then studied in combination with each of the other foods with which it may be used in practice. It is necessary to proceed from the simple to the complex mixtures in these studies. Ultimately it is hoped that diets can be planned which will promote the optimum of physiological well being, and therefore lead to the optimum in physical development, length of life, and the preservation of youthful characteristics.

In publishing this series of papers dealing with the studies of the dietary properties of several types of food mixtures, several new observations will be pointed out. The interpretation of the results is based upon more careful and thorough observations than have hitherto been described in any similar studies. They include not only the rate and extent of growth, the fertility, and success in rearing of young, but also the period of life up to and including the onset of old age with its characteristic changes.

Since animal tissues have a very prominent place in the diet of man in most parts of the world, it is of great moment to understand the value of these with respect to each of the essential dietary factors. Our knowledge of the nutritive qualities of animal tissues is still very incomplete. Watson and Hunter<sup>4</sup> showed that rats fed exclusively on muscle meats suffered severe malnutrition. Liver has, however, found great favor as a food for young fish in hatcheries. It has been shown<sup>5</sup> that lard does not contain appreciable amounts of fat-soluble A, whereas fats extracted from a glandular organ (pig kidney or cod testicle) are a good source of it.<sup>6</sup> Liver and kidney have been shown<sup>7</sup> to be a good source of both fat-soluble A and water-soluble B, whereas muscle tissue is very poor in both.

Heart, a variety of muscle, was found on the other hand to contain sufficient of both fat-soluble A and water-soluble B to support growth for a time at least in young rats.<sup>7</sup>

<sup>4</sup> Watson, C., and Hunter, A., *J. Physiol.*, 1906, xxxiv, 111.

<sup>5</sup> McCollum, E. V., and Davis, M., *J. Biol. Chem.*, 1913, xv, 167. Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1913-14, xvi, 423.

<sup>6</sup> McCollum, E. V., and Davis, M., *J. Biol. Chem.*, 1915, xx, 641.

<sup>7</sup> McCollum, E. V., and Davis, M., *J. Biol. Chem.*, 1915, xxi, 179. Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1917, xxxii, 309; 1918, xxxiv, 17.



McCollum<sup>8</sup> pointed out that the mineral content of animal tissues such as muscle and glandular organs resembles that of seeds of plants sufficiently to indicate that it would not prove satisfactory as a source of inorganic elements in animal nutrition.

No data exist showing the comparative values of the proteins of the several kinds of animal tissues. Osborne and Mendel<sup>7</sup> have shown that growth takes place in young rats restricted to 18 per cent of protein derived solely from liver, kidney, muscle, or brain. This is a liberal amount, and is far above the plane of intake necessary for the support of normal growth in the rat when the quality of the protein is good.

There is also reason to inquire into the possibility of the presence of toxic substances in the glandular organs. The presence of such powerful physiological stimulants as thyroxin and adrenalin in the thyroid and suprarenal glands, respectively, makes them unfit for human or animal food. We have been informed that the Eskimos do not eat the liver of the reindeer. This fact may perhaps be accounted for by the absence of a gall bladder in this species, and consequent high content of bile, which would give it a bitter flavor. The Eskimos are said not to eat the liver of the polar bear, to avoid that of a certain species of seal, and to believe the liver of the dog poisonous. The liver is concerned with so many types of transformations of organic substances which have their origin in metabolism that it seemed possible that certain of these may be present in sufficient amounts to be detrimental to one who eats freely of it. Similar consideration might lead one to inquire whether the kidney of an animal may contain sufficient amounts of certain metabolic products as to render it undesirable as a food.

The glandular organs are rich in cell nuclei and consequently yield considerable amounts of purines when metabolized. These ultimately are converted in great measure into uric acid. There are conditions of perverted metabolism in man in which the excretion of uric acid and urates is interfered with and doubtless under such circumstances liver or kidney should not be eaten. There would seem little reason, however, why these organs should not be eaten by healthy persons as adjuvants to the diet. They possess

<sup>8</sup> McCollum, E. V., *The newer knowledge of nutrition*, New York, 1918.

dietary properties as distinct from those of muscle tissue as the leaves of plants do in contrast to the seeds.

In regions such as Labrador and Newfoundland where the diet of the habitants consists essentially of wheat flour, molasses, fish, meats, tea, and raisins, beri-beri and scurvy are common. A condition popularly called night-blindness is also of frequent occurrence among these people. On such a diet one would suspect the danger of developing xerophthalmia and apparently this is the case. The successful treatment of night-blindness has recently been reported by the administration of cod liver oil. This oil is a good source of fat-soluble A and is very effective in the cure of xerophthalmia.

Fresh liver is rich in fat soluble-A, water-soluble B, and water-soluble C, the protective dietary factors for ophthalmia, beri-beri, and scurvy, respectively. It should be easily possible to eradicate these dietary diseases in such regions as Labrador and Newfoundland by the use of fish livers as food. It is strange indeed that the natives of these regions have failed to discover the value of a by-product of their fishing industry which would serve in a great measure to correct the conspicuous faults in their diet.

For the purpose of comparing the biological values of the proteins of kidney, liver, and muscle, we have followed the procedure described by McCollum, Simmonds, and Parsons<sup>9</sup> of feeding the tissues singly as the sole sources of protein at planes so as to introduce 9 per cent of protein into the food mixture. They were supplemented with respect to all other factors so as to make a satisfactory diet, with the possible exception of the protein moiety. Lots 2475, 2476, and 2474, Chart 1, represent experiments of this type. Such experiments make it possible to compare the proteins of these animal tissues with those of a number of combinations of cereal and other seed proteins with milk which we have previously studied.<sup>10</sup> It has been shown that normal growth is secured when the diet contains 9 per cent of protein of high biological value. If much less than this content of protein is furnished by the food mixture, even when the protein is excellent, the growth falls distinctly below the curve of normal expectation.

<sup>9</sup> McCollum, E. V., Simmonds, N., and Parsons, H. T., *J. Biol. Chem.*, 1919, xxxvii, 155.

<sup>10</sup> McCollum, E. V., and Davis, M., *J. Biol. Chem.*, 1915, xx, 415.

When proteins of several types such as those of cereals, legume seeds, and milk were compared in this manner it was found that the proteins of milk were superior to those of most seeds of plants yet examined. The seed which furnishes proteins nearest to milk in value for conversion into body proteins during growth is wheat, which therefore stands first among the cereals in value. From the records of Chart 1, which show the effects of feeding kidney, liver, and muscle proteins respectively at 9 per cent of the food mixture, it is apparent that the proteins of these substances are scarcely superior to those of certain cereals, especially wheat. We base this conclusion on the growth of young rats restricted to one of these sources of protein at the critical 9 per cent level. Normal growth is not secured with wheat proteins fed below this plane of intake.

This result is very surprising indeed. It seems best explainable on the assumption that the patterns on which the proteins of the muscle are constructed differ very decidedly from that of the liver or kidney, and presumably from the glandular organs as a class. The nutritive needs of the body involve the replacement of tissues of both organ and muscle types. Apparently glandular organs or muscle as the sole source of nitrogen in the food fails to serve for efficient transformation into new body proteins during the symmetrical growth of the body tissues. They are complete but their transformation into body proteins cannot be very effectively accomplished (Chart 1).

We have shown in a previous communication that the cereal grain proteins do not in general make good each other's deficiencies or enhance to any great extent each other's biological values when two of them are combined.<sup>9</sup> Rye and flaxseed meal proteins form a notable exception. These, when combined in certain proportions, form a mixture which is distinctly superior to the proteins of either component alone. The supplementary value of one protein for another depends on the yields by each of those indispensable amino-acids which are present in each of the sources in smallest amounts. We shall show in one of the following papers that kidney, liver, and muscle proteins have much greater values as supplements to the cereal proteins than the cereal or legume proteins have, with few exceptions, among themselves. The data in the succeeding paper illustrate the importance of animal tissues in the food supply

when the diet consists mainly of such vegetable products as do not yield a mixture of proteins having a high biological value. For the special purpose of enhancing the quality of the protein in the diet they have the highest value. It must be kept in mind, however, that their use does not correct the mineral deficiencies of a cereal, tuber, and legume seed diet, and that when muscle meats are used as food they have little effect in raising the content of fat-soluble A in the resulting mixtures. In no instance, therefore, will one of these types of animal tissue supplement a cereal, tuber, and fleshy root type of diet so as to make it highly satisfactory.

Our observations on the rats described in these experiments do not show any definite evidence of injury to the animals as the result of being fed excessively high protein diets. These diets were, however, essentially complete and fairly well proportioned as regards all factors other than protein. It is not justifiable to generalize from these results that such a high protein intake is safe for man. Our animals were not kept to determine the possible span of life or the time of appearance of the signs of senility, owing to the necessity of temporarily vacating the room in which the animals were kept. It is the custom for people in the United States to derive a high protein diet, when such is taken, in great measure from muscle meats, fish, poultry, eggs, and legume seeds. Menus containing such high protein foods will only in exceptional cases be completely supplemented by other constituents of the diet. The evil effects often attributed to excessive protein consumption may now with some confidence be attributed in many instances to faults in the composition of the diet in factors other than protein. Further studies are required to demonstrate the relative merits of diets of high and low protein contents when other factors are of comparable value in the two cases. We shall discuss this phase of nutrition on the basis of carefully planned experiments on animals in a later communication.

There are some very interesting and important instances of successful nutrition among people who have lived almost exclusively upon a diet very rich in protein and derived from foods of animal origin. We are indebted to Mr. Vilhjalmur Stefansson for the information that previous to about 1850 dental caries were very rare or absent from Iceland. During the last half of the 19th century cases have gradually become more and more common

until today infected teeth are perhaps as common there as in most parts of the United States. No carious teeth were found among 96 skulls disinterred by Stefansson from a cemetery in Iceland dating from the 9th to the 13th centuries. These skulls are now in the Peabody museum at Harvard University and have been described by Hooton.<sup>11</sup> The diet of the Icelanders previous to about 1850 consisted essentially of milk, mutton, fish, and fowl, but in some parts of the island they ate the eggs of wild birds. The only vegetable food eaten regularly was carrageen moss, but potatoes and turnips were eaten to some extent. The teeth of the natives and their general health were excellent as long as this diet was taken. The deterioration of the teeth apparently began about the time when cereals and sugar were regularly imported into Iceland as sources of food.

The teeth of the primitive Eskimo were excellent. The younger generation in northern Alaska, whose diet is derived in a large measure from cereal grain products, canned foods, and muscle meats, similar to what would be purchased in a grocery store in the United States, has poorly calcified teeth which are often carious.

We have collected numerous observations on the effect of dietary faults on the quality of the skeleton in the rat. These data make it clear that most profound differences in the extent of calcification and density of the deposited calcium phosphate can be effected by such faults as are found in the cereal, tuber, and muscle meat type of diet.

The diet of the primitive Eskimo was very rich in protein but it was at least fairly satisfactory with respect to other factors. It consisted of muscle tissue and fat as the principal components, but all blood was carefully saved and eaten, and the glandular structures were regarded as dainties of especial delicacy. In addition, they regularly ate bone marrow and chewed the softer parts of bones, such as ribs and the epiphyses of the long bones. Such a selection of tissues suffices for the satisfactory nutrition of the rat and produces the fine physical development seen in the Carnivora such as the lion, tiger, jaguar, etc. The deterioration of the teeth of the Eskimo which occurred simultaneously with the modification of the diet due to contact with the white man is in

<sup>11</sup> Hooton, E. A., *Am. J. Physical Anthropol.*, 1918, 1, 53.

harmony with what we have been led to expect as the result of experimental studies of the types of combinations of ordinary foods which enter into the diet of man and animals in different parts of the world. The cereal and muscle meat diet or its equivalent, the bread, meat, and potato type of diet, is in all probability the cause of the deterioration of the teeth of the present generation of "civilized" Eskimos as it is among the people of the United States and Europe.

#### CONCLUSIONS.

The kidney, liver, and muscle of the ox contain proteins which, when they serve as the sole source of nitrogen, and are fed singly as the sole source of protein, but completely supplemented with respect to all necessary factors other than protein, are shown to possess about the same biological value as those of the wheat kernel.

There is no distinct evidence of toxicity in either muscle, kidney, or liver tissue when fed at planes of intake sufficiently high to introduce from 35 to 70 per cent of protein into the diet.

The first limiting factor in the kidney, liver, and muscle tissue is a lack of calcium. It is also necessary to add sodium chloride in order to insure prolonged well being. Carnivorous man and animals secure their sodium chloride by eating blood, and calcium by eating bone. Liver and kidney contain an abundance of fat-soluble A and of water-soluble B, and when fresh and raw, of water-soluble C. Muscle tissue is very deficient in these factors but does not entirely lack any one of them. Kidney proteins appear to have higher biological value than those of the other animal tissues yet studied.

It has been our custom for years in preparing experimental diets to thoroughly grind the several components of the food and to make a uniform mixture from which the constituent parts cannot be picked out by the animals. In all cases iodine was given once a week in the form of potassium iodide-iodine in the drinking water, which was distilled. The liver, kidney, and steak, except when the contrary is stated, were steamed in a sterilizer until thoroughly cooked, subsequently dried, and ground. Practically all visible fat was removed from both organs and muscle. The curves presented in the charts are typical representatives of a group of four to six animals which composed each experimental group.

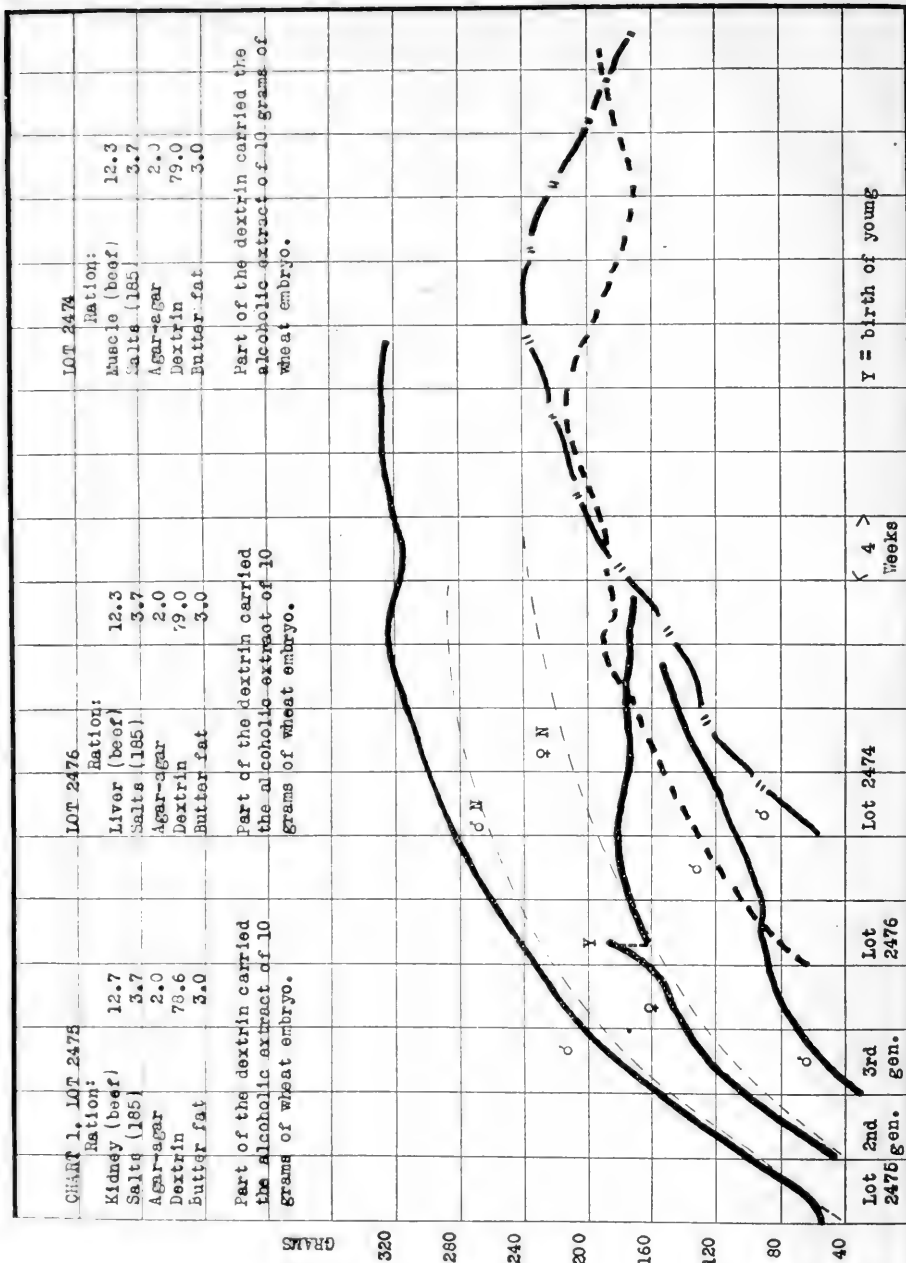
*Chart 1.*—The curves in this chart illustrate the growth of young rats fed diets containing 9 per cent of protein derived solely from beef kidney, liver, and muscle, respectively. The inorganic additions were of a character which completed, at least in a fairly satisfactory manner, the mineral content of these animal tissues. There can be no doubt that kidney proteins have a somewhat higher biological value than those of liver. Liver proteins, surprising as it may seem, have not been found in our experiments to be superior to those of muscle (steak).

In Lot 2475 there were two females. One of these died in parturition. The other at the age of 4 months had one litter of four young and successfully reared them. She died from unknown cause about 40 days after weaning her young. Two of her daughters grew up and produced one and three litters of young (a total of seventeen), respectively. Of these ten were successfully weaned.

Two granddaughters of the female described in the original experimental group were kept  $6\frac{1}{2}$  months on the family ration, and although they appeared to be in good condition, neither proved fertile. The rats fed the kidney ration did not exhibit early signs of aging. In this respect they were superior to the groups fed diets containing comparable amounts of liver and of muscle.

The records of these experimental groups fed 9 per cent of protein from kidney, liver, and muscle, respectively, all other factors being more or less satisfactorily adjusted, show them to be typical examples of nutritional instability. In Lot 2475 on the kidney diet, each succeeding generation was inferior to its parents. Lack of uniformity of vitality among individuals of the same group or family is observed with striking frequency in animals whose diets fall but little below the quality necessary to maintain the vigor of the species unimpaired throughout successive generations.

It might be suggested that the failure of the animals fed liver to develop more satisfactorily, was due to the presence of toxic substances in this organ, which performs the function of degrading numerous foreign and poisonous substances derived from metabolism and absorption from the alimentary tract and elsewhere. The records of Chart 2 show clearly that this is not the case.





Neither of the two females fed the steak diet had any young, although they appeared to be well nourished and were kept under observation during more than 12 months.

Three females on the liver diet were somewhat undersized, aged early, and never had any young, although they were kept under observation for more than a year. In the case of both the liver and muscle diets this was apparently due to the proteins from these sources not being of sufficiently good quality to make 9 per cent of protein from these sources sufficient to promote well being at the optimum, since in these diets all other factors were corrected.

*Composition of Salt Mixture 185.*

	<i>per cent</i>
NaCl.....	0.173
MgSO <sub>4</sub> (anhydrous).....	0.266
NaH <sub>2</sub> PO <sub>4</sub> + H <sub>2</sub> O.....	0.347
K <sub>2</sub> HPO <sub>4</sub> .....	0.954
CaH <sub>4</sub> (PO <sub>4</sub> ) <sub>2</sub> H <sub>2</sub> .....	0.540
Fe citrate.....	0.118
Ca lactate.....	1.300

*Chart 2.*—The rats in these experiments were fed food mixtures which were satisfactorily constituted except for possible shortage of water-soluble B and fat-soluble A, which in each case were derived entirely from 25 per cent of kidney, liver, or muscle, respectively. The growth curves and fertility of the animals on the kidney and liver diets show that these amounts were satisfactory as sources of fat-soluble A and water-soluble B. In these diets the protein of the animal tissues was supplemented with 9 per cent of casein. Chart 4 shows, however, that 20 per cent of either kidney or liver suffices as the sole source of protein for normal growth, reproduction, and rearing of young, for the fourth generation on the kidney diet appeared to have normal vitality. The rats of the fourth generation on the liver diet were inferior, although they were successfully weaned.

Two females on the kidney diet, Lot 2163, had collectively four litters (fourteen young), all of which were reared. Two of the daughters were maintained during 10 months on the diet on which their mothers had lived. One remained sterile, the other had a single litter (five young) at about 4 months of age and never became pregnant afterwards. The young were in good condition when weaned.

CHART 2. LOT 2162

Ration:  
Liver (beef) 25.0  
Casein 9.0  
NaCl 1.0  
KCl 1.0  
CaCO<sub>3</sub> 1.5  
Dextrin 62.5

LOT 2163

Ration:  
Kidney (beef)  
Casein  
NaCl  
KCl  
CaCO<sub>3</sub>  
Dextrin

LOT 2160

Ration:  
Period 1  
Cooked muscle  
Casein 25.0  
NaCl 9.0  
KCl 1.0  
CaCO<sub>3</sub> 1.0  
Dextrin 1.5  
62.5

Period 2

5 per cent of butter fat  
replaced part of hextrin.

LOT 2161  
Ration:  
Periods 1 and 2 like Lot 2160  
but raw muscle replaced the  
cooked round steak.

GRAMS

360

320

280

240

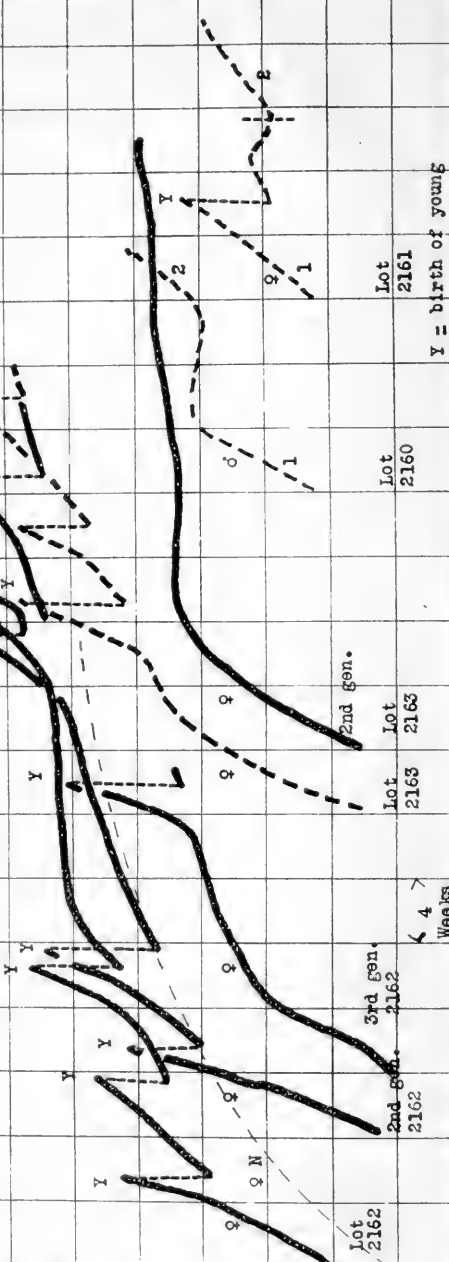
200

160

120

80

40



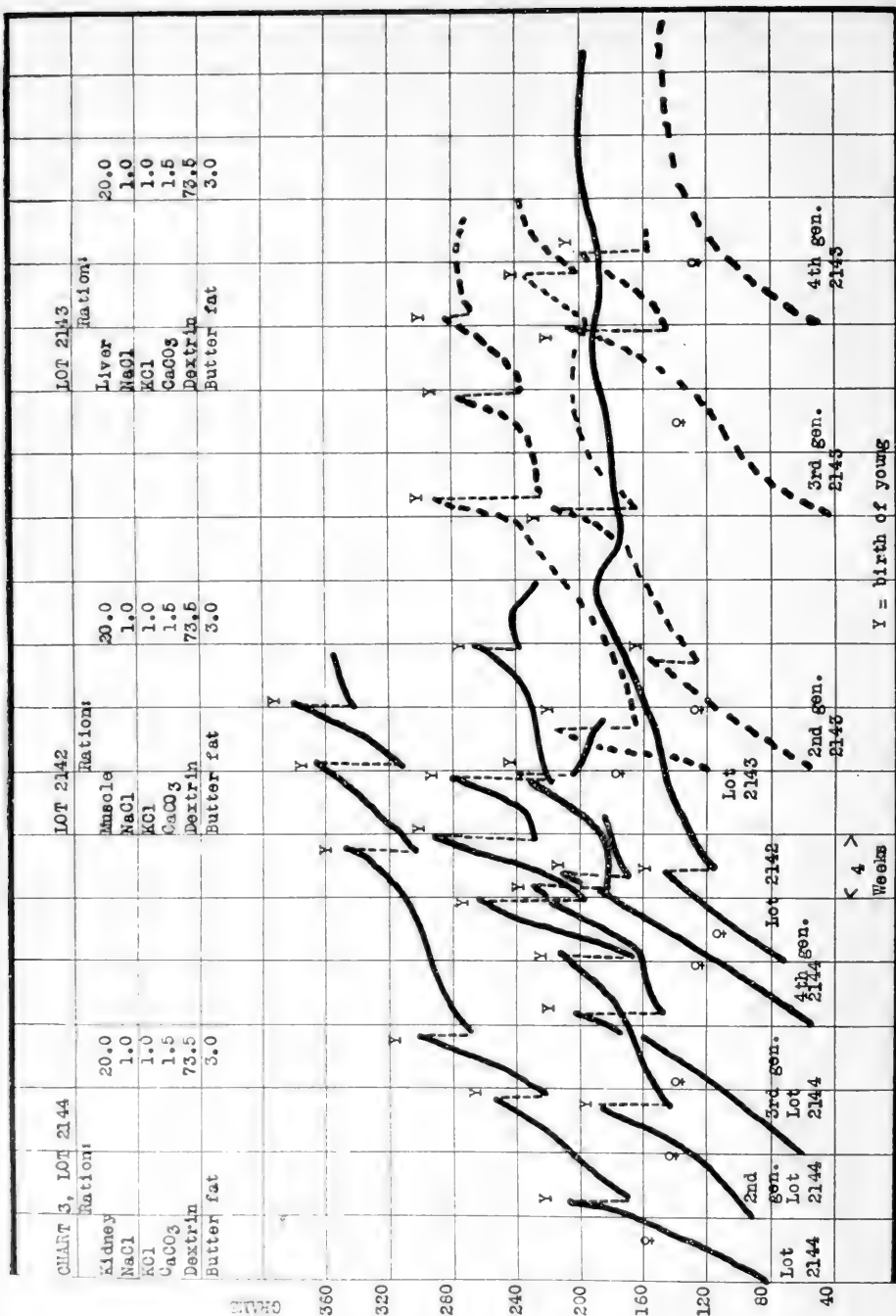
There were three females raised on the liver diet (Lot 2162). They had collectively fifty-six young (nine litters), of which thirty-three were weaned. The young which died became lethargic just before weaning time and died in this condition. We have at present no explanation for this peculiar behavior.

Lots 2160 and 2161 were fed diets comparable to Lots 2162 and 2163, except that the factors, fat-soluble A and water-soluble B, were entirely derived from 25 per cent of muscle tissue (cooked and raw beefsteak, respectively). In both cases there was failure of growth after about 4 weeks. After a period of suspended growth during which one female in Lot 2161 had a litter of four young which were eaten by the mother shortly after birth, 5 per cent of butter fat was added to the diet of each group. In both cases there was a marked response to growth following this addition. This demonstrates that 25 per cent of round steak does not contain sufficient fat-soluble A to meet the needs of the young rat. Lot 2142, Chart 3, shows clearly that 20 per cent of steak does not furnish sufficient water-soluble B for the normal nutrition of young rats during growth.

The records in this chart show clearly the remarkable difference between the glandular organs as compared with muscle tissue in respect to their content of both the factors, fat-soluble A and water-soluble B. This supports the view which we have repeatedly stated, that the dietary properties of a substance can be fairly accurately predicted from a knowledge of their biological function.

*Chart 3.*—The three groups of animals whose curves are shown in this chart were fed diets comparable in all respects, except that the sole source of water-soluble B and protein was 20 per cent of the dry matter in the diet in the form of kidney, liver, and muscle, respectively. A comparison of these curves with those of Chart 1 shows that the protein furnished by this proportion of liver, kidney, and steak, respectively, suffices to support normal growth when other factors in the diet are properly adjusted.

The animals fed the kidney ration, Lot 2144, were markedly superior to the other two groups. Representatives of four successive generations were grown upon this diet as their sole source of water-soluble B, and with no evidence of deterioration. In the original group there were three females. These produced collectively ten litters (forty young) but only six individuals were



successfully weaned. The mothers destroyed their young soon after birth.

Two daughters of the females just described were kept on the family diet. They had together six litters (forty young) and weaned only four individuals altogether. Here again the mortality was the result of cannibalistic tendencies in the mothers. But one granddaughter of the original group was kept on the family diet. She grew normally and had six young (two litters) and successfully weaned all of them. One great granddaughter was brought up on the diet. She had three young (one litter) and weaned them successfully. With optimum nutrition these litters would have been two or three times as large as those produced.

It might be suggested that perhaps the content of water-soluble B in this diet was below the optimum, and that for this reason the nutrition of the nervous systems of the rats restricted to this diet was faulty, and that the tendency to destroy the young was an expression of an abnormal psychology analogous to the psychosis observed in beri-beri or pellagra. The fact that the third and fourth generation females in this family were less cannibalistic than their mothers and grandmothers militates somewhat against this view.

Lot 2143 derived all its antineuritic factor and protein from 20 per cent of liver in the diet. This was equivalent to 14.4 per cent of protein. The growth curves were normal. In the first group fed this diet there were two females. These had forty-six young (eight litters) and of these but twenty were weaned. Two second generation females had eighteen young (four litters) and weaned ten of them. Two third generation females had thirteen young (two litters) of which they weaned six. One fourth generation female was kept 5½ months, when she died, never having had any young. None of these animals in any generation was kept to an advanced age. The young of the rats fed the liver diet were somewhat undersized but appeared to be vigorous.

Lot 2142 was fed a diet comparable to the others described in this chart, but with all protein and water-soluble B derived from 20 per cent of muscle (round steak). These animals were markedly inferior both as regards growth and fertility to those fed kidney or liver at the same plane of intake. Two females were kept to the age of 15 months, an age which usually marks the end of

fertility in the rat. One had a single litter of eight young but they died when about 2 weeks old. The other remained sterile. The rats in this group were still in good nutritive condition after a year on the diet. This chart, like the other records in this paper, shows clearly the superiority of glandular organs over muscle tissue as sources of water-soluble B.

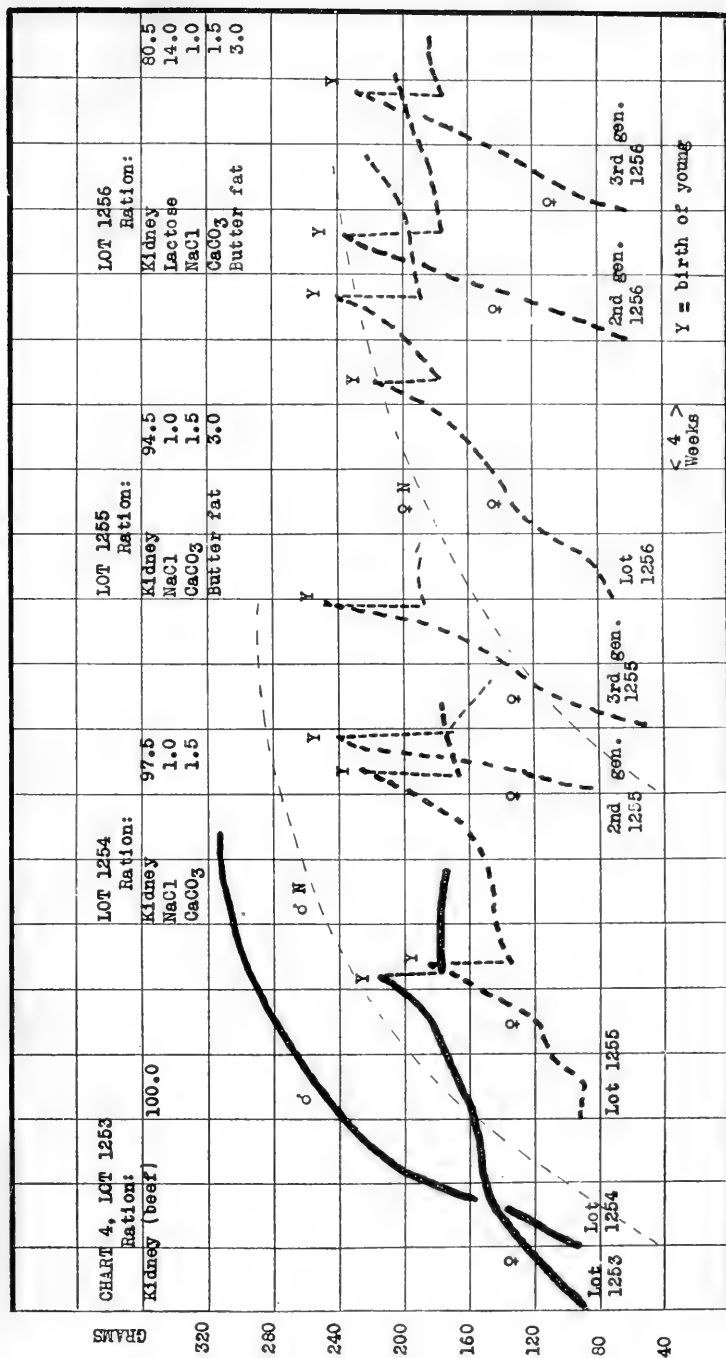
*Chart 4.*—These records show the behavior of young rats fed cooked dry beef kidney as the sole food, Lot 1253. Lot 1254 shows the growth curves of rats fed 97.5 per cent of kidney supplemented with sodium chloride and calcium carbonate. Lot 1255 was fed kidney, 94.5 per cent, supplemented with sodium chloride, calcium carbonate, and butter fat. Lot 1256 was fed a diet like that of Lot 1255 except that 14 per cent of the kidney was replaced by lactose. This last ration was designed to show whether lactose would tend to modify the bacterial flora of the alimentary tract. No beneficial effects of the modification were apparent.

The rats restricted to cooked dried kidney as their sole food grew in a fairly normal manner. Two females had collectively fourteen young (a litter each), five of which were weaned. One of these young developed abnormal ribs suggestive of rickets. None of these young grew up.

It is very remarkable that young rats confined to a diet of kidney could develop so successfully and reproduce and rear young. This tissue is very poor in calcium, and yields a great excess of acid on being metabolized. The protein content of this diet was not far from 71 per cent, yet because all essential food factors except calcium were so abundant, the animals were able to tolerate this deficiency and the abnormal protein content remarkably well.

Lot 1254 contained two females, one of which became pregnant and died in parturition. The other had a tumor which became so large that she was chloroformed.

There was but one female in Lot 1255. She had sixteen young (two litters) of which nine were weaned. Two second generation females had collectively seventeen young (a litter each) of which ten were weaned. Two third generation females had each a litter of young (seventeen) of which twelve individuals were weaned. The young appeared to be well developed, but were always greasy from their food. They drank much water. The cage had a strong odor. The protein content of the ration of Lot 1254 was 69 per



cent; that of Lot 1255, 67 per cent; of Lot 1256, 57 per cent. The results show that the rat is capable of growing and remaining in a state of health on a diet comparable to that of the strictly carnivorous animals. At least among the carnivorous animals of the Arctic regions the proportion of protein in the diet is not necessarily excessive at all times. Mr. Stefansson has informed us that it is not unusual for travelers to come upon a seal that has been killed and skinned, the subcutaneous layer of fat eaten, and the remainder of the carcass left practically untouched. The polar bear evidently prefers fat to protein as a source of energy.

Lot 1256 contained two females. They had collectively twenty-two young (two litters each) of which fifteen were weaned; two second generation females had together nineteen young, and weaned fourteen of them. One third generation female had one litter of six young and weaned them all.

*Chart 5.*—The diet of the four groups of rats whose curves are shown in this chart was in all instances comparable to those in Chart 4, the only difference being the substitution of liver for kidney in the diets of Lots 1277, 1278, 1279, and 1280.

Lot 1277, which was confined to liver as its sole food, grew very little and died from 4 to 6 months after being confined to the diet.

Lot 1278 was fed liver supplemented with sodium chloride and calcium carbonate. On this diet growth was approximately normal. The group contained three females. One of these had twelve young (two litters) and weaned eleven of them. The other two remained sterile. One second generation female remained sterile.

Lot 1279 was fed liver supplemented with sodium chloride, calcium carbonate, and butter fat. Lot 2162, Chart 2, shows that even 25 per cent of liver as the sole source of fat-soluble A furnishes a sufficient amount of this factor.

There were three females in this group. They had thirty-four young (six litters) and weaned thirty-one of them. One second generation female grew up on the diet, and had six young in one litter. She weaned four of these. One third generation female in this family had a litter of six and weaned them all. The reproduction records of this group were distinctly better than those of Lot 1278, whose diet was identical except for the 3 per cent of but-





ter fat. We cannot explain the reason for the superiority of Lot 1279.

Lot 1280 was like Lot 1279 except that 14 per cent of lactose replaced a like amount of liver. This did not exert any noticeably favorable effect on the well being of the animals. Two females each had a litter (collectively fourteen young) and weaned thirteen individuals. One second generation female had one litter of eight young and weaned seven of these. The young in Lots 1279 and 1280 appeared to be strong and in good condition but showed in all cases a peculiar condition which we have observed occasionally in abnormal animals; *viz.*, a wet and stained area around the urethral orifice. This not infrequently occurs in poorly nourished animals.

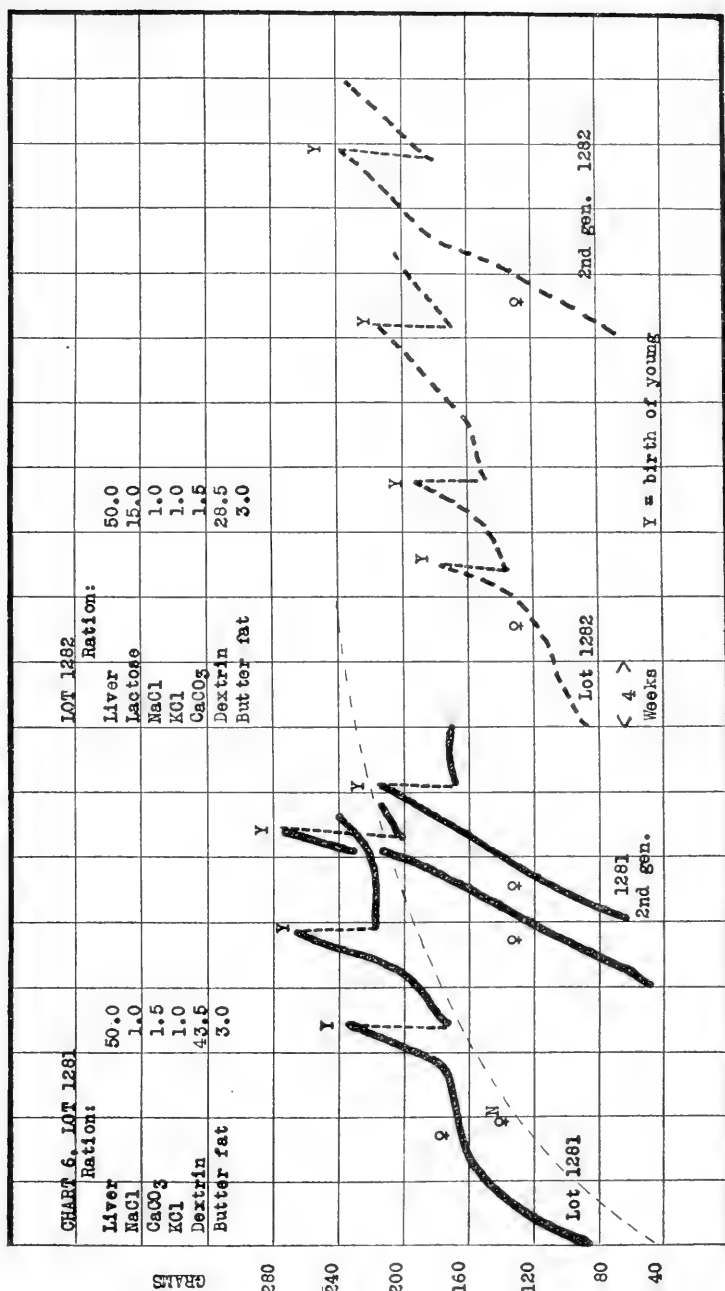
*Chart 6.*—The animals whose records are shown in this chart were fed a diet made adequate in all respects as far as could be judged, and with the protein derived solely from 50 per cent of dry beef liver. The protein content of the diet was about 35 per cent.

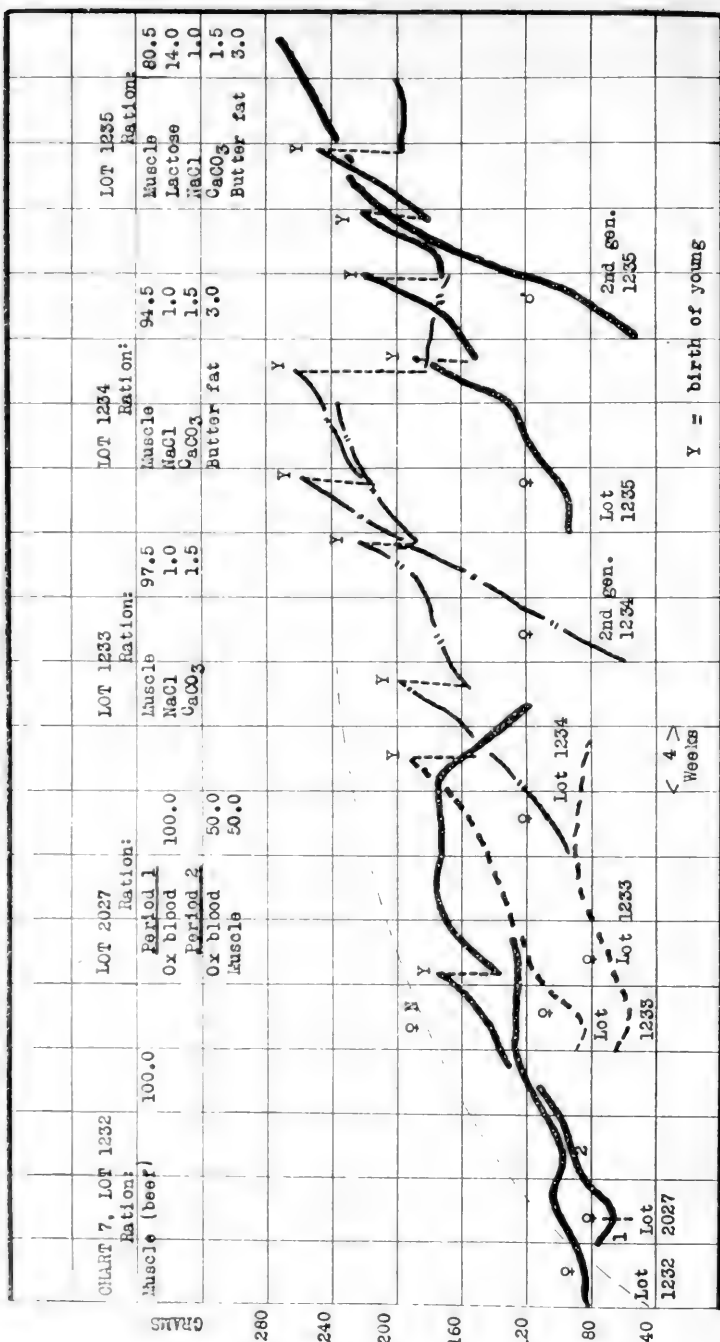
Lot 1281 contained three females. These had collectively thirty-five young (five litters) and weaned twenty-two. Two second generation females had one litter each, collectively nineteen young, of which number they weaned seventeen. These young appeared to be very well nourished.

Lot 1282 contained two females. These collectively had five litters (thirty-four young). They weaned thirty-one of these young.

Two second generation females were restricted to this diet. One had a litter of six, which she successfully weaned. The other female remained sterile. The young were apparently well nourished but badly urine-stained. There was no evidence that the inclusion of lactose benefited the animals.

*Chart 7.*—The animals whose records are shown in this chart were fed either muscle tissue or blood, with and without certain purified food additions. Lot 1232 was restricted to a diet of cooked, dried, beef muscle. They were able to grow very slowly, but remained very much undersized, and died early. None of this group had any young. Two of the rats showed, toward the end of their lives, distinct signs of xerophthalmia, due to the lack of fat-soluble A in muscle tissue.





Lot 2027 was fed dried ox blood as their sole food in Period 1. They declined rapidly on this and after 2 weeks were changed to a diet of muscle meat 50 per cent and dried blood 50 per cent. On this diet they were able to grow at a rate approximately half the normal. A female in this group had a single litter of four young, which she destroyed within a few days. All these animals showed early signs of old age.

Lot 1233 was fed cooked dried muscle (beefsteak) supplemented with sodium chloride and calcium carbonate. They grew but little better on this diet than on muscle alone, but one female had a litter of three young, which she destroyed soon after birth. Two other females remained sterile on this diet. That fat-soluble A was the limiting factor in this diet is shown by the records of Lots 1234 and 1235.

Lot 1234 was fed muscle, sodium chloride, calcium carbonate, and butter fat. They not only grew in an approximately normal manner, but were fairly fertile and had moderate success in the rearing of their young. Two females had together twenty young (three litters) of which sixteen were weaned. One second generation female was kept on the diet. She had one litter of five young and weaned them all. The young were somewhat inferior. Further studies are necessary to determine what modifications of this diet are necessary to secure greater fertility and higher vitality in the young.

Lot 1235 was fed a diet of beefsteak supplemented with sodium chloride, calcium carbonate, butter fat, and 14 per cent of lactose. They were not superior in vigor, fertility, or success in rearing young to Lot 1234 which had more beefsteak in place of the lactose. Two females had thirty-two young collectively (five litters) of which twelve were weaned. Since these were all males no further reproduction records were secured.

*Chart 8.*—The animals whose records are shown in Chart 8 derived their protein and water-soluble B from 50 per cent of cooked, dried, muscle tissue (beefsteak). The protein content of the diet of Lot 1236 was about 35 per cent. The growth curves approximated the average and the animals appeared to be in a satisfactory state of nutrition.

There were two females in Lot 1236. Together they had thirty-four young (five litters) and weaned twenty-four of them. One



daughter of one of these mothers was kept on the diet. She had one litter of eight young and weaned them all. Two of her daughters grew up on the diet and each had a litter, together thirteen young, and weaned but three of them. The young in most cases appeared normal. They were not urine-stained as many were on the liver diets.

Lot 1237 differed from Lot 1236 in that 15 per cent of lactose replaced an equivalent amount of dextrin. There was no evidence that this carbohydrate exerted any beneficial effect over dextrin.

Two females in this group had together thirty young (six litters) and successfully weaned them all. Two of the second generation females had each a litter (collectively fifteen young), of which eleven young were weaned. One third generation female had a litter of seven young and weaned them all.

*Chart 9.*—These records show the histories of three groups of young rats fed diets in which all protein and antineuritic factors were derived from raw muscle tissue (round steak). Lot 2056 was restricted in the first period to raw round steak as the sole source of nutriment. They grew no better on this than on cooked steak (Chart 7, Lot 1232). In a second period sodium chloride and calcium carbonate were added and these caused a slight response with growth for a time. The diet was too poor in fat-soluble A to admit of much growth. This is seen in the record of Lot 2058.

Lot 2058 was fed a diet containing 50 per cent of raw, dried muscle (beefsteak) supplemented with sodium, potassium, calcium, chlorine, and fat-soluble A in butter fat. The diet contained 43.5 per cent of dextrin. On this ration growth was normal and the animals were apparently in good nutritive condition. Three females each had a litter of young (collectively twenty-two) of which twenty were reared. Two second generation females each had a litter (fourteen young) and reared them all.

There was a period between the ages of 18 and 28 days when the young of the second generation appeared lethargic. They recovered later and appeared to be as alert as rats on the cooked steak. This was probably due to the effects of eating so much raw meat while still in a very immature condition.

Lot 2057 had a diet like that of Lot 2058 except that 15 per cent of dextrin was replaced by a like amount of lactose. There was





no noticeable benefit from this modification of the diet. There were two females in this group. They had collectively twenty-three young (four litters) of which twenty-two were weaned. No young have as yet been secured from any of the daughters. These records when compared with those of Chart 8 indicate that no noticeable difference exists in the nutritive value of raw and cooked steak. Although steak is very poor in water-soluble B, there was a sufficient amount of it in 50 per cent of cooked steak to permit young rats to grow up and rear young.



## SUPPLEMENTARY PROTEIN VALUES IN FOODS.

### II. SUPPLEMENTARY DIETARY RELATIONS BETWEEN ANIMAL TISSUES AND CEREAL AND LEGUME SEEDS.

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(Received for publication, March 21, 1921.)

In a former publication<sup>1</sup> we have shown that the proteins of the navy bean do not markedly supplement those of the maize kernel so as to improve their quality for the support of growth. This means that the proteins of this cereal and legume seed are both deficient in some of the same indispensable amino-acids. Our earlier studies also indicated that the cereal proteins do not have much of a supplementary value among themselves.<sup>2</sup> In a later paper of this series we shall present more records bringing out the extent to which the proteins of the cereal grains and the legume seeds enhance each other's values for the nutrition of the rat. The data presented in the charts show the nutritive values of the protein mixtures resulting from combinations of the cereal grains and legume seeds with several representative animal tissues.

We recently pointed out the surprising fact that when muscle tissue, liver, or kidney, serves as the sole source of protein in the diet, all other factors in which have been made satisfactory by suitable additions, the quality of the amino-acid mixture for transformation into body proteins during growth is no better than that of proteins derived from grains.<sup>3</sup> It has always been

<sup>1</sup> McCollum, E. V., Simmonds, N., and Pitz, W., *J. Biol. Chem.*, 1917, xxix, 521.

<sup>2</sup> McCollum, E. V., Simmonds, N., and Parsons, H. T., *J. Biol. Chem.*, 1919, xxxvii, 155.

<sup>3</sup> McCollum, E. V., Simmonds, N. and Parsons, H. T., *J. Biol. Chem.*, 1921, xlvii, 111.

accepted as a fact, hardly in need of experimental demonstration, that these animal proteins have exceptionally high biological values. Thomas came to this conclusion as the result of experimental data obtained on a full grown man as a subject. The periods of observation were very short, and a milk diet was used in the foreperiods which vitiated the results because of the supplementary effect on the foods being studied. Thomas' experimental data represent digestibility and absorbability of proteins rather than biological value for transformation into body tissue.<sup>4</sup>

TABLE I.

Chart.	Lot.	Source of protein.	Growth.	Fertility.	Mortality.
2	2193	$\frac{2}{3}$ wheat, $\frac{1}{3}$ kidney.	Excellent.	High.	Medium.
	2192	$\frac{2}{3}$ soy beans, $\frac{1}{3}$ kidney.	Fair.	Very low.	High.
3	2479	$\frac{2}{3}$ barley, $\frac{1}{3}$ kidney.	Good.	High.	Medium.
4	2195	$\frac{2}{3}$ rolled oats, $\frac{1}{3}$ kidney.	Fair.	Medium.	"
5	2194	$\frac{2}{3}$ maize, $\frac{1}{3}$ kidney.	"	"	High.
	2191	$\frac{2}{3}$ navy beans, $\frac{1}{3}$ kidney.	"	Low.	Low.
6	2190	$\frac{2}{3}$ peas, $\frac{1}{3}$ kidney.	"	"	High.
	2196	$\frac{2}{3}$ rye, $\frac{1}{3}$ "	Good.	Medium.	"

Of all the combinations of proteins from the two sources which we have studied, wheat and kidney proved to have the highest biological value. We have tested only one mixture of proteins from these sources; *viz.*, that in which a seed furnished two-thirds and kidney one-third of the total. Since in many cases growth was essentially normal, the basis of judgment in the comparison of the values of protein mixtures was the variation in fertility and infant mortality.

The combination of navy bean and kidney proteins forms a mixture having a higher biological value than any other animal tissue and legume seed which we have studied. Soy bean and kidney form a better protein mixture than soy bean with liver or soy bean and muscle.

The results of our studies show that the proteins of kidney, liver, and muscle have remarkable values for the enhancement of the proteins of some of the cereal grains, and improve the legume seed proteins to an appreciable degree. Kidney, liver, and muscle proteins have without exception supplemented those of the cereal grains more satisfactorily than they do those of the legume seeds. There are marked differences in the efficiency of the sup-

<sup>4</sup> Thomas, K., *Arch. Physiol.*, 1909, 219.

plementary relations between kidney proteins and those of several cereal grains which make it possible for us to differentiate between their values in the case of the different combinations. The relative values of the several combinations of vegetable proteins with those of kidney with which we have experimented in this series may be illustrated by tabulating the seeds in the order of their efficiency for growth, when two-thirds of the protein is derived from the seed and one-third from kidney.

Wheat—barley—rye—oat—maize—soy bean—pea—navy bean.

There is no marked differentiation between the kidney and oat as contrasted with the kidney and maize combinations. These are distinctly inferior to the combinations of kidney with barley, wheat, or rye. There is little difference in the biological values of the mixtures of proteins derived from peas or the two kinds of beans studied. These legume seeds combined with animal tissues are inferior to similar combinations of animal tissues with cereals. Table I shows in condensed form the effects of feeding kidney proteins as supplements to certain vegetable proteins on growth, fertility, and infant mortality.

Among the several combinations of cereal and legume seed proteins with muscle tissue which we have studied, wheat is decidedly the best. Growth and fertility were remarkable, but the infant mortality was high. Muscle tissue is distinctly less effective than kidney as a supplement to the proteins of the seeds used, except in the case of wheat. The following order represents the biological values of combinations of seed with muscle in which the seed furnished two-thirds of the total protein and the muscle one-third. The values are arranged in a descending scale.

Wheat—oat—barley—maize—pea—navy bean—soy bean.

It is not possible to differentiate from our data between the values of navy bean and soy bean proteins combined with muscle. Table II shows in condensed form the effects on growth, fertility, and infant mortality of feeding muscle proteins as supplements to the proteins of several seeds.

Liver proteins supplement those of the cereal grains and legume seeds in degrees which differ sufficiently to make possible rather decided contrasts in several cases. In general, liver proteins

enhance those of the seeds somewhat better than do those of muscle, with the single exception of the combination, wheat and muscle. The seeds which we have studied may be arranged in the following order to illustrate the supplementary efficiency of liver proteins for each, the best combination being on the left and decreasing from left to right.

Barley—rye—wheat—oat—maize.

TABLE II.

Chart.	Lot.	Source of protein.	Growth.	Fertility.	Mortality.
7	2186	$\frac{2}{3}$ wheat, $\frac{1}{3}$ muscle.	Excellent.	High.	High.
8	2189	$\frac{2}{3}$ rye, $\frac{1}{3}$ " "	Very good.	"	"
	2184	$\frac{2}{3}$ navy beans, $\frac{1}{3}$ muscle.	Poor.	Low.	"
9	2188	$\frac{2}{3}$ rolled oats, $\frac{1}{3}$ " "	Good.	High.	"
	2477	$\frac{2}{3}$ barley, $\frac{1}{3}$ muscle.	"	Low.	"
10	2187	$\frac{2}{3}$ maize, $\frac{1}{3}$ " "	Fair.	"	"
	2183	$\frac{2}{3}$ peas, $\frac{1}{3}$ " "	"	"	"
	2185	$\frac{2}{3}$ soy beans, $\frac{1}{3}$ muscle.	"	"	"

Wheat and muscle gave an excellent growth curve and high fertility, but apparently because of a slight inferiority of this protein mixture as compared with certain others, the infant mortality was high. Wheat and muscle appear to form a protein mixture superior to rye and muscle. The growth curves of rats confined to a protein supply derived from rolled oats and muscle were better than the curves of those fed rolled oats and kidney. The latter, however, were more successful in fertility and in rearing young than the former.

It is not possible from our data to decide between the values of the combinations of liver proteins with those of the legume seeds, peas, navy beans, and soy beans. A study of the records presented here, showing the effects of seed and meat diets, supplemented with respect to all factors other than protein, on the life histories of individual animals and on the family histories of successive generations confined to monotonous food mixtures, cannot fail to impress one with the fact that observations on the growth curves of a group of animals to maturity do not afford sufficient evidence to enable one to arrive at safe deductions concerning the quality of an experimental diet. Table III shows the effect on growth, fertility, and infant mortality, of feeding liver proteins as supplements to the proteins of several seeds.

There are many who believe that if the diet affords sufficient calories and considerable variety, satisfactory nutrition will be assured. This may be far from true. So long as the selection does not include a variety of types of foods of unlike dietary values variety does not insure safety in any great degree. No matter how many cereals, tubers, and muscle meats such as steak, ham, chops, roasts, etc., may be taken, the diet will prove to be inadequate. The inclusion of milk or leafy vegetables goes far toward

TABLE III.

Chart.	Lot.	Source of protein.	Growth.	Fertility.	Mortality.
11	2478	$\frac{2}{3}$ barley, $\frac{1}{3}$ liver.	Good.	High.	Medium.
	2178	$\frac{2}{3}$ soy beans, $\frac{1}{3}$ liver.	Poor.	Low.	High.
12	2180	$\frac{2}{3}$ maize, $\frac{1}{3}$ liver.	Fair.	Medium.	"
13	2181	$\frac{2}{3}$ rolled oats, $\frac{1}{3}$ liver.	Good.	High.	"
14	2182	$\frac{2}{3}$ rye, $\frac{1}{3}$ liver.	Very good.	"	"
	2176	$\frac{2}{3}$ peas, $\frac{1}{3}$ "	Fair.	Low.	"
15	2179	$\frac{2}{3}$ wheat, $\frac{1}{3}$ "	Good.	High.	"
	2177	$\frac{2}{3}$ navy beans, $\frac{1}{3}$ liver.	Fair.	Low.	"

Rollled oats and liver formed a better protein mixture than rolled oats with kidney or muscle. This could not be discerned from an inspection of the growth curves or the records of fertility, but came to light through the comparison of the infant mortality in the three groups.

Barley and liver made a better protein mixture than barley and muscle, but not quite so good as barley and kidney. Rye and liver also proved superior as a source of protein to mixtures of this cereal with either muscle or kidney.

Navy beans with liver form a better protein mixture than combinations of this seed with muscle, but not quite so good as with kidney.

making good deficiencies of such a diet in inorganic constituents and fat-soluble A. Fresh fruits and uncooked salad vegetables have a unique place in the diet as sources of the antiscorbutic factor water-soluble C.

The substitution at frequent intervals of ham for chops will not tend to insure safety in nutrition. So large a part of the food supply of man in the temperate and warmer regions of the world is derived from cereals and other seed grains, tubers, and edible roots, that in seeking variety in food attention should be fixed upon the importance of milk and the leafy vegetables suitable for

greens, salads, etc., since these are so constituted as to correct the deficiencies of cereals, tubers, and roots.<sup>5</sup>

In examining the records of the experimental animals described in this paper it should be kept in mind that in studying the combinations of proteins from animal and vegetable sources, we have in all cases added butter fat to furnish fat-soluble A and the inorganic salts necessary to correct the mineral deficiencies. The results do not, therefore, represent the supplementary effect of one of these foodstuffs for another except in respect to the protein factor.

The results of these studies form a demonstration of the value of animal tissues as constituents of the diet, when the latter contains as much cereal products as is usually the case in America and Europe at the present time. At the same time it must be remembered that the animal tissues are by no means complete supplements for a cereal and legume seed diet.

#### SUMMARY.

1. The proteins of kidney, liver, and muscle are remarkably effective as supplements for the proteins of cereals. There are demonstrable differences in the extent to which these animal tissues enhance the values of certain of the cereals. Thus, kidney, liver, and muscle are about equally effective as supplements to wheat. Maize proteins are less effectively supplemented by kidney, liver, or muscle than wheat, barley, or rye.

2. The three animal tissue proteins studied were found to have a supplementary relation to pea, navy bean, or soy bean proteins, but these combinations are very inferior to similar combinations of animal tissues with cereal grains as a source of protein.

3. Our results demonstrate that the proteins of kidney, liver, or muscle are more valuable for transformation into body tissues when combined with cereal proteins, than when each is fed as the sole source of amino-acids in the diet.<sup>3</sup>

4. Either muscle or glandular substance fails to supplement effectively the mineral deficiencies of cereals or legume seeds. The glandular organs are good sources of fat-soluble A.

<sup>5</sup> McCollum, E. V., Simmonds, N., and Parsons, H. T., *J. Biol. Chem.*, 1919, xxxviii, 113.



*Chart 1.*—These curves show typical records of growth and reproduction of rats fed from an early age on diets in which the sole source of protein was a cereal or legume seed. The amount of seeds was adjusted in each case so as to give the diets a protein content of 9 per cent. Such additions of butter fat and inorganic salts were made in each case as to correct dietary deficiencies other than protein of these seeds.

The list of cereals here discussed includes wheat, maize, rolled oats, barley, and the legumes, peas, navy beans, and soy beans.

Among these seeds wheat stands first in quality of protein when fed as the sole source of this factor. Oat and maize proteins have approximately the same value, but we have gained the impression that oat proteins are slightly superior. None of the legume seeds is at all satisfactory as the sole source of protein in the diet at the plane of intake of 9 per cent of the food mixture. This fact is brought out by the curves of Lots 2376, 2510, and 2368. On adding casein in a second period growth was accelerated in each case.

No young have been secured on diets of this type in which proteins were derived entirely from a legume seed or from maize. The fertility of rats fed oats to furnish 9 per cent of protein was so far below that of animals fed wheat protein in similar amounts as to indicate that the latter were in a distinctly better state of well being. Even in the case of the wheat diet the protein was not sufficiently good to permit of much success in rearing young. A few young could be reared by mothers which had grown up and were maintained on the oat diet containing 9 per cent of protein.

These curves are presented as a basis of comparison for the records in the remaining charts described in this paper. These derive their content of protein (9 per cent) in each case from two sources. The object in each case is to compare the biological value of mixtures of proteins in stated proportions as compared with the values of each source when fed alone.

*Chart 2.*—Lot 2193 was fed a diet from soon after weaning, which derived its protein content of 9 per cent from wheat and cooked dry kidney. All other factors in the diet were adequately supplemented. The wheat furnished 6 per cent and the kidney 3 per cent of the protein of the diet. Growth was normal in this group. The state of nutrition of these animals was much superior

CHART 1, LOT 2481

Ration:

Wheat 90.0  
NaCl 1.0  
CaCO<sub>3</sub> 1.5  
Dextrin 4.5  
Butter fat 3.0

LOT 2484

Ration:

Roller oats 60.0  
NaCl 1.0  
CaCO<sub>3</sub> 1.5  
Dextrin 34.5  
Butter fat 3.0

LOT 3483

Ration:

Barley 75.0  
NaCl 1.0  
CaCO<sub>3</sub> 1.5  
Dextrin 19.5  
Butter fat 3.0

LOT 619

Ration:

Maize 90.0  
NaCl 1.0  
CaCO<sub>3</sub> 1.5  
Dextrin 4.5  
Butter fat 3.0

LOT 2376

Ration:

Period 1  
Pens 40.8  
NaCl 1.0  
CaCO<sub>3</sub> 1.5  
Dextrin 53.7  
Butter fat 3.0  
Period 2  
Casein 10 per cent  
replaced part of  
dextrin.

LOT 2368

Ration:

Like Lot 2376  
with navy beans  
replacing peas.

LOT 2310

Ration:

Period 1  
Soy beans 24.9  
Salts (1.5) 3.7  
NaCl 1.0  
CaCO<sub>3</sub> 1.5  
Dextrin 65.9  
Butter fat 3.0  
Period 2  
5 per cent of casein  
replaced part of  
dextrin.

Wt. lbs

240

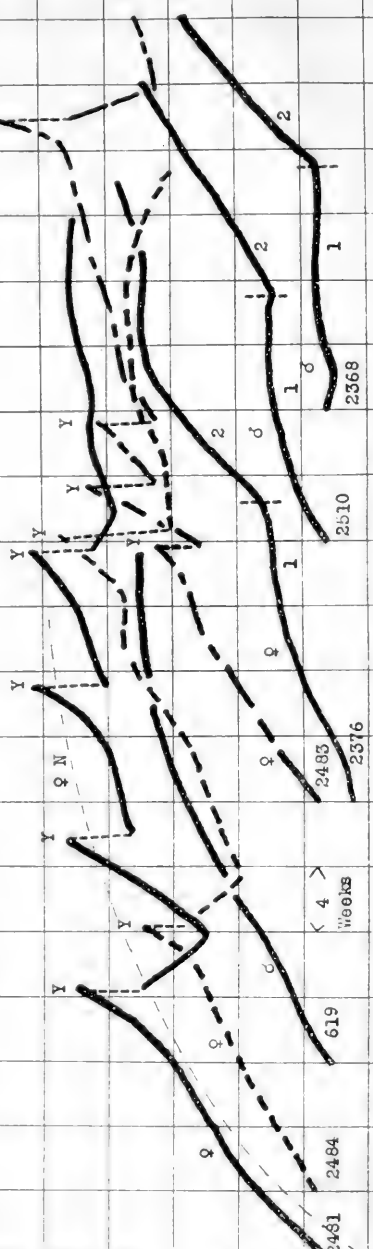
200

160

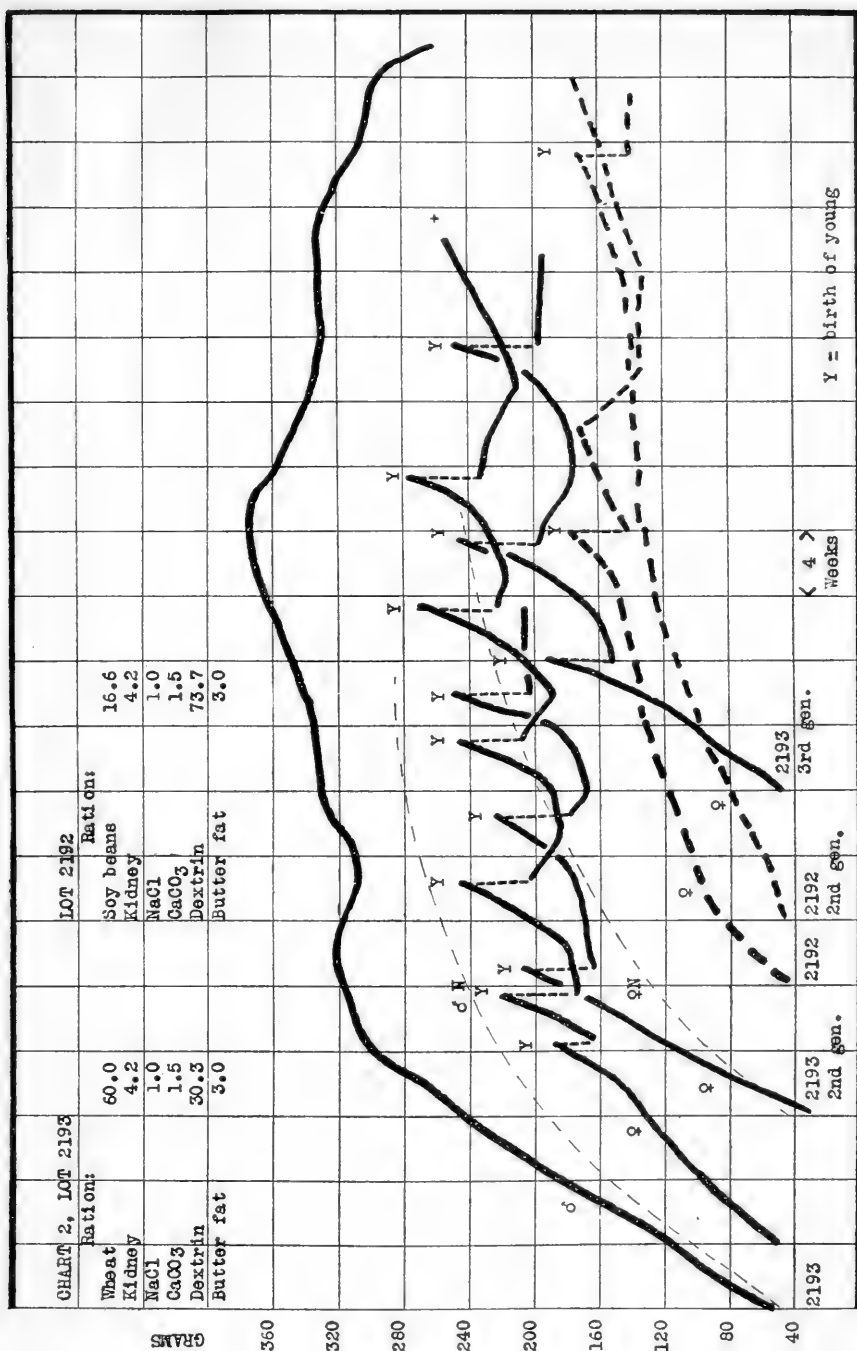
120

90

40



Y = birth of young



to those fed wheat as the sole source of protein when the content of the latter was limited to 9 per cent. Those rats which grew up on 9 per cent of wheat protein (Lot 2481, Chart 1) were as old looking at 14 months as were those in Lot 2193 at 18 to 20 months. Indeed, the animals of Lot 2193 were in such good condition that they compared favorably in appearance and in fertility with other experimental groups fed much higher protein intakes.

There were two females in this group. They had collectively seventy-nine young (eleven litters) and weaned seventy of them. On a diet comparable in all respects but with all its 9 per cent of protein derived from wheat we observed 50 per cent mortality of the young. A somewhat higher protein content in the diet of Lot 2193 would apparently have shortened the nursing periods, which were longer than normal, and would have lowered the infant mortality. Four females in the second generation had seventy-five young (twelve litters) and weaned forty-nine of them. Two third generation females had forty-nine young (eight litters) and weaned thirty-six. The mortality of the young in this group was due entirely to destruction by the mothers. Frequently the young were bitten and injured or killed when approaching the end of the nursing period.

One cannot say that animals, which had a diet containing enough protein to enable them to grow at the normal rate to maturity and to be as fertile as were these, were suffering from protein starvation. Yet it was due to the low protein intake (of the quality here used) which exerted a peculiar influence on the psychological reactions of the mothers and made them attack their young even after they were 2 or 3 weeks old. In rats in our colony which have highly satisfactory diets it is extremely rare that a mother attacks her offspring or indeed those of another. We not infrequently isolate among our stock animals two or three pregnant females in the same cage. They then care for their young in a common nest and nurse their own young and those of the other mothers promiscuously and without perversion of the maternal instinct.

Lot 2192 was fed a diet in which the protein was furnished by soy beans and beef kidney. The diet contained 9 per cent of total protein, two-thirds of which came from soy beans and one-third from kidney. All the other factors were satisfactorily supplemented. Growth was slow and the animals never reached full

adult size. Their growth was much better, however, than that of rats restricted to the same amount of protein entirely from soy beans (Chart 1, Lot 2510).

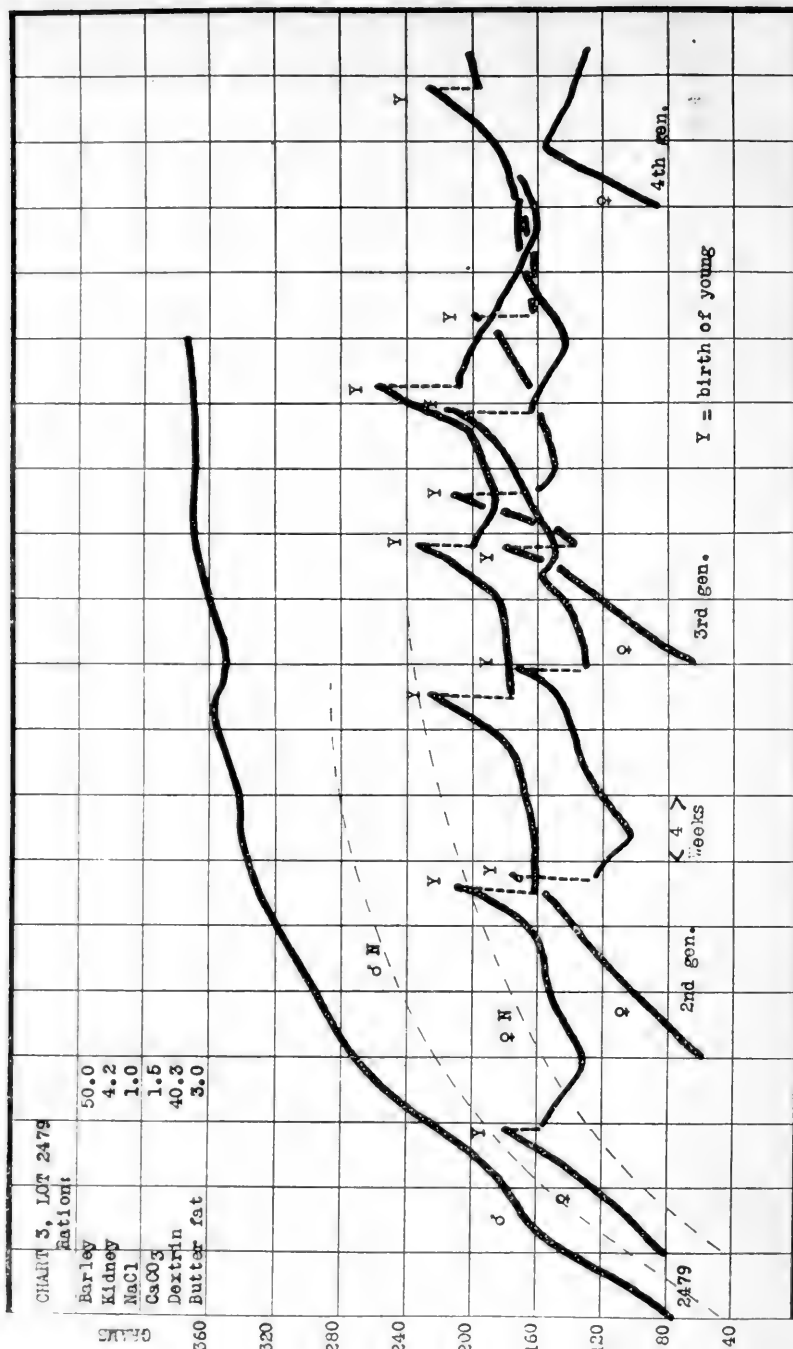
Two females grew up on this diet. One remained sterile. The other had nine young (two litters) and weaned three. Two second generation females remained sterile. The animals in this group were very old looking at 15 months of age. It is evident that 9 per cent of protein from these sources is not satisfactory for normal milk production.

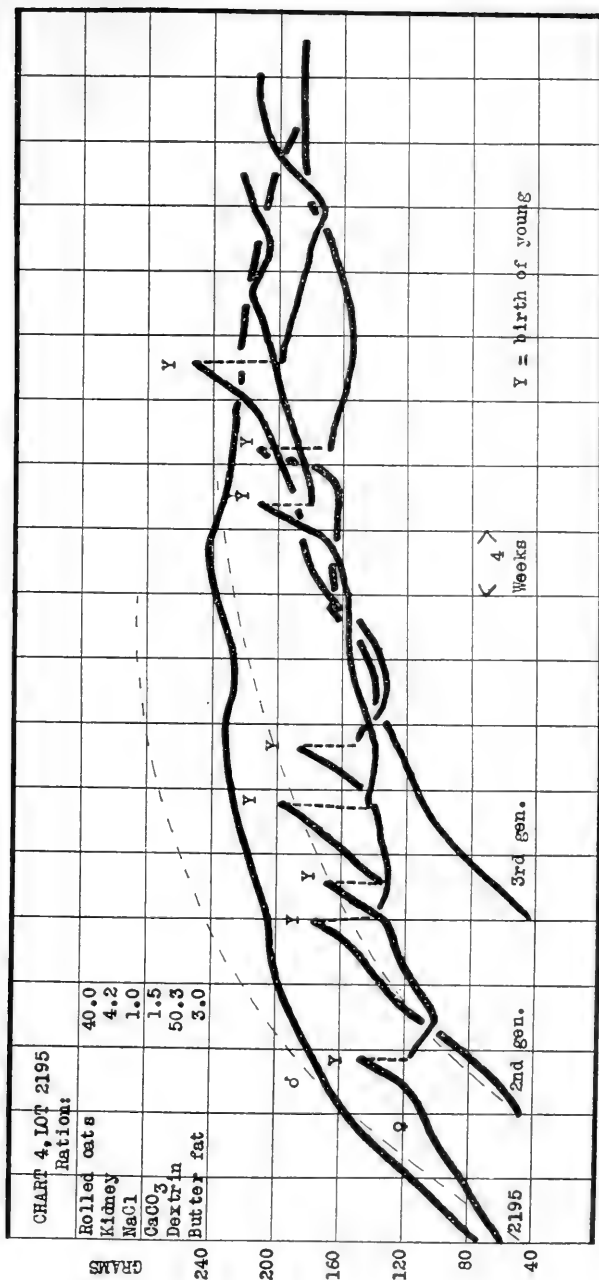
*Chart 3.*—Lot 2479 was fed a diet in which the protein was derived from barley and kidney. The barley furnished two-thirds and the kidney one-third of the total protein. The growth was more satisfactory than any we have observed in rats fed 9 per cent of barley protein only (see Chart 1, Lot 2483).

Two females which grew up on this diet had collectively forty-five young (seven litters) and weaned thirty-one. One female in the second generation had nineteen young (three litters) and weaned eighteen. Two third generation females had forty-three young (seven litters) and weaned nineteen. One fourth generation female was restricted to the diet but she failed early and died at the age of 110 days. These animals were in good condition at the age of 15 months, when they were discarded.

*Chart 4.*—Lot 2195 was fed a diet in which the protein was furnished entirely by rolled oats and kidney. The oats furnished two-thirds and the kidney one-third of the total protein. Their records were very much better than those of Lot 2484 (Chart 1). This is shown especially in the fertility and infant mortality of the two groups. Lot 2195 was somewhat undersized but of good appearance. They were not in such good condition as those which grew up on the wheat and kidney diet (Lot 2193, Chart 2).

Two females grew up on this diet. One of these had twenty young (four litters) and weaned ten. The other died soon after giving birth to her first litter of six young. Two second generation females had thirty-seven young (six litters) and weaned twenty-three. One third generation female had one litter of five and weaned them all. These young were in good condition, but the nursing periods were long. The animals in this group were old looking at about 16 months.





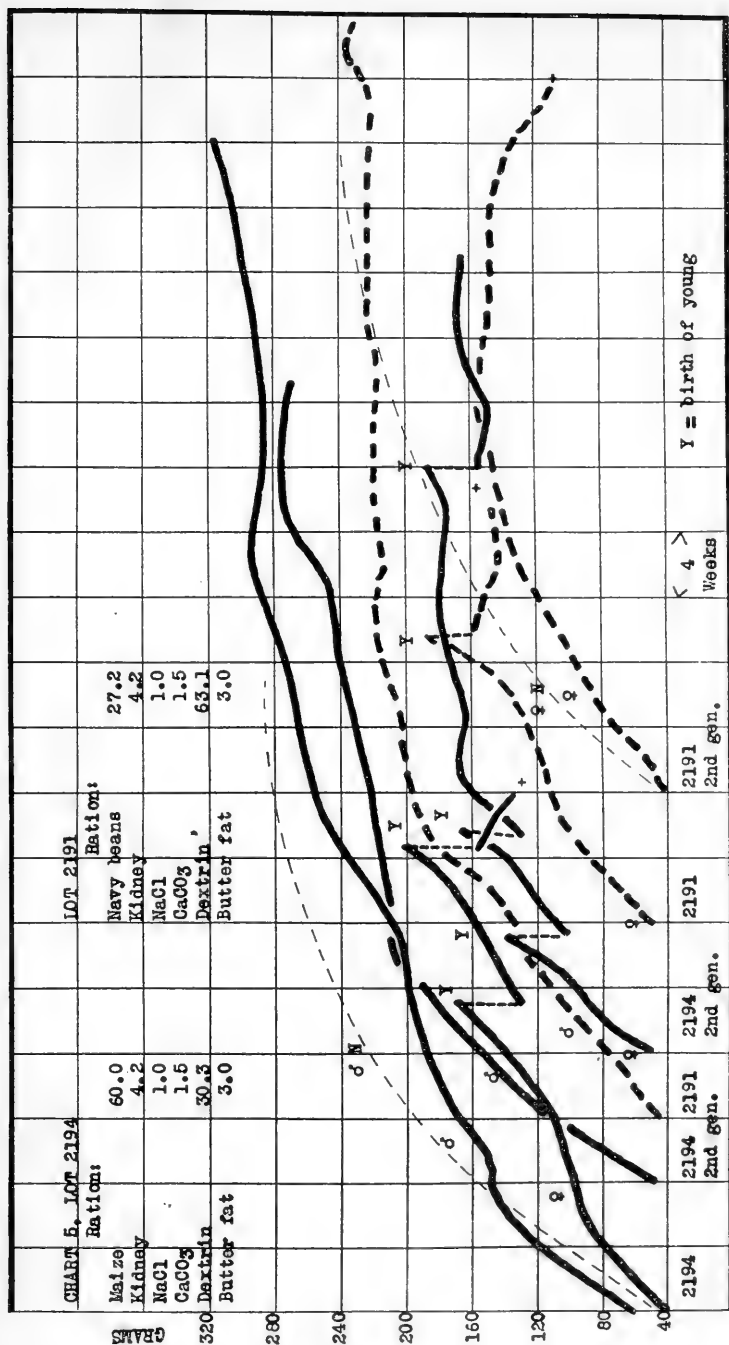
*Chart 5.*—Lot 2194 was fed a diet, the protein of which was derived entirely from maize and kidney. The maize furnished two-thirds of the total protein and the kidney one-third. All other factors were satisfactorily supplemented. The growth curves were much better than were those of Lot 619 (Chart 1), which had a diet containing 9 per cent of maize protein alone. The animals of Lot 2194 on the maize and kidney proteins were never in good condition, but did not look very aged until about 18 months. Three females which grew up on this diet had thirty-one young (six litters) and weaned fifteen. They were only in fair condition and were small for their ages. One second generation female grew up on the diet and had fifteen young (three litters). She weaned four of her third litter. The other young she destroyed soon after they were born.

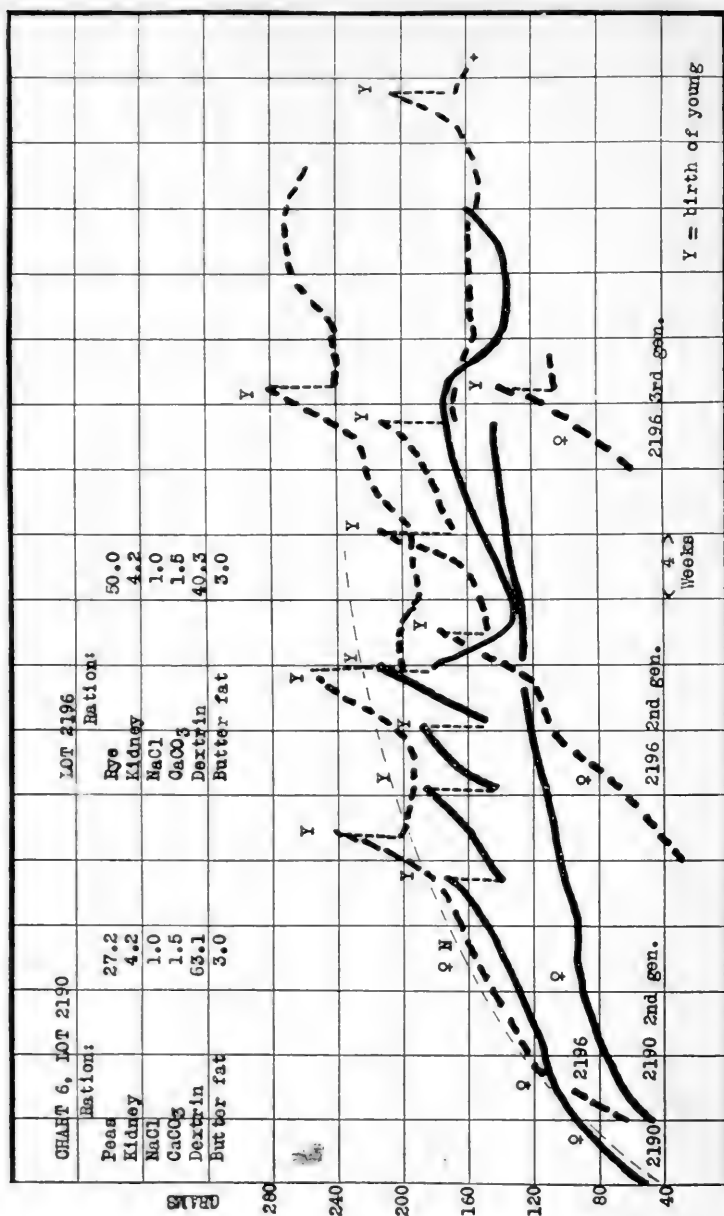
Lot 2191 was fed a diet containing 9 per cent of protein, two-thirds of which was furnished by navy beans and one-third by kidney. All factors other than protein were satisfactorily supplemented. A diet exactly similar but with 9 per cent of protein derived solely from navy beans supports almost no growth (Lot 2368, Chart 1). The combination of navy beans and kidney is therefore vastly superior to bean protein alone. These animals looked very old at about 12 months.

One female in this group had one litter of five young and weaned four, but they were very small. At 42 days of age the four weighed but 147 gm. A litter of four nursing a normally fed mother should reach a weight of about 350 gm. at this age. One other female in this group remained sterile. Two second generation females were restricted to the diet but neither one had any young although their growth curves were much better than those of rats confined to 9 per cent of navy bean protein alone (Lot 2368, Chart 1).

*Chart 6.*—Lot 2190 was fed a diet containing 9 per cent of protein, two-thirds of which was furnished by peas and one-third by kidney. All other factors were satisfactorily supplemented. The animals grew much better than a group confined to a similar diet with 9 per cent of pea protein as its sole source of nitrogen (Lot 2376, Chart 1). This shows that there is a greatly heightened value in the combination of proteins used in this diet as compared with pea protein alone. The rats fed this ration were somewhat







undersized, but appeared fairly well nourished in early life. They appeared senile when about 12 months of age.

One female in this group had twenty-four young (four litters) and weaned four of her fourth litter. The other young were destroyed by the mother while very young. Another female remained sterile. Three second generation females were confined to the diet until they were 12 months old, but none of them had any young.

Lot 2196 derived its sole protein from rye and kidney. The rye furnished two-thirds and the kidney one-third of the total. Except for the amount and quality of the protein the diet was well constituted. Although the growth of these animals was excellent and the fertility fairly high, the mortality of the young was very high. These rats were in good condition in early life but looked very old at about 16 to 18 months.

Five females on this diet had collectively seventy-three young (fourteen litters) and weaned thirty-one. Many of these were destroyed by the mothers soon after birth. One female died after the first litter, and a second died after her second litter from undetermined causes. Two second generation females had thirty-one young (seven litters) and weaned nine. One third generation female had a litter of six.

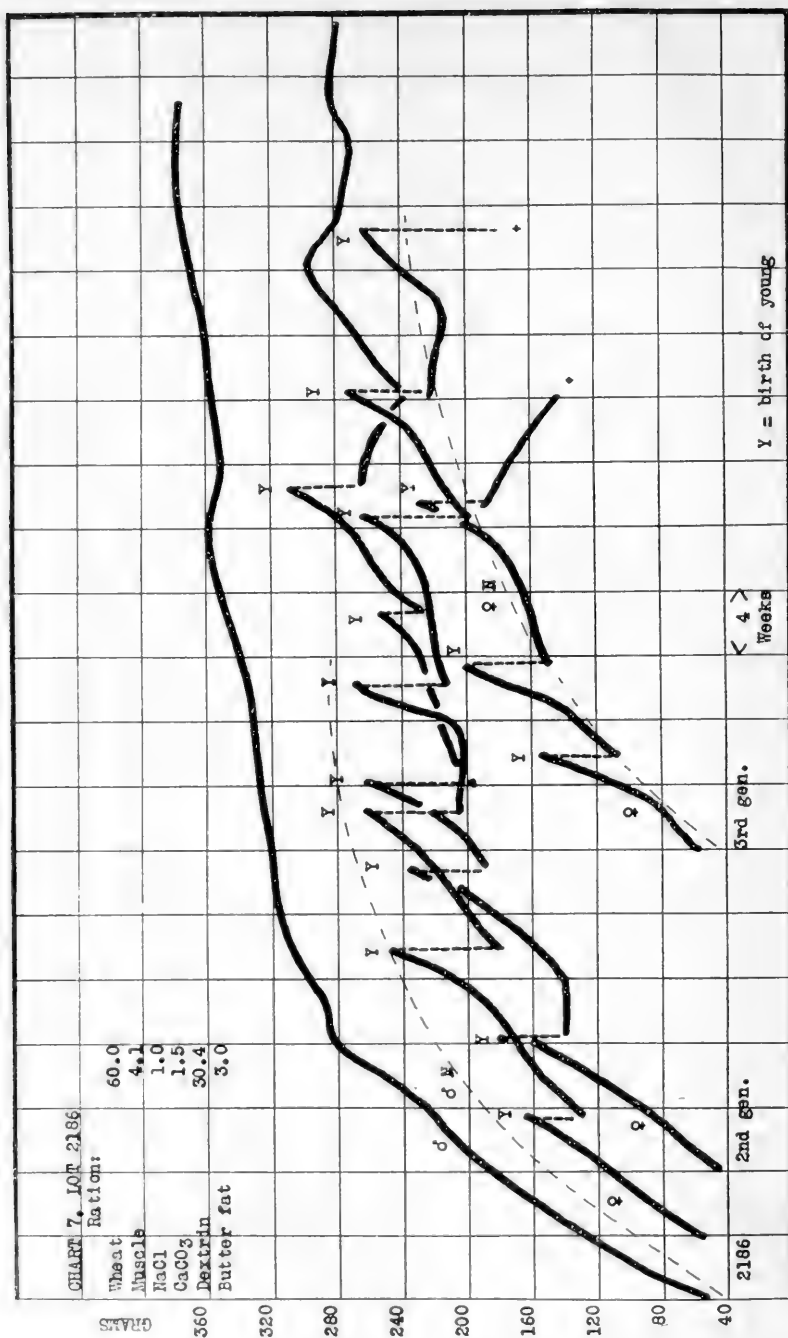
Our investigations show that cereal grain proteins are distinctly better supplemented by kidney proteins than are those of the legume seeds. The order of effectiveness in this supplementary relation is as follows:

Wheat—barley—rye—oat—maize.

There is no marked difference in the value of peas, soy beans, and navy beans in their supplementary relation to kidney proteins.

*Chart 7.*—Lot 2186 was fed a diet comparable to those discussed in Charts 2 to 6 inclusive, except that muscle tissue (beefsteak) replaced kidney as a supplemental source of protein for a seed. This diet contained 9 per cent of protein, two-thirds of which was furnished by wheat and one-third by beef muscle. Deficiencies in inorganic salts and fat-soluble A were made good by suitable additions.

The growth curves of this group, considering that the food mixture contained but 9 per cent of protein, are remarkable. All the

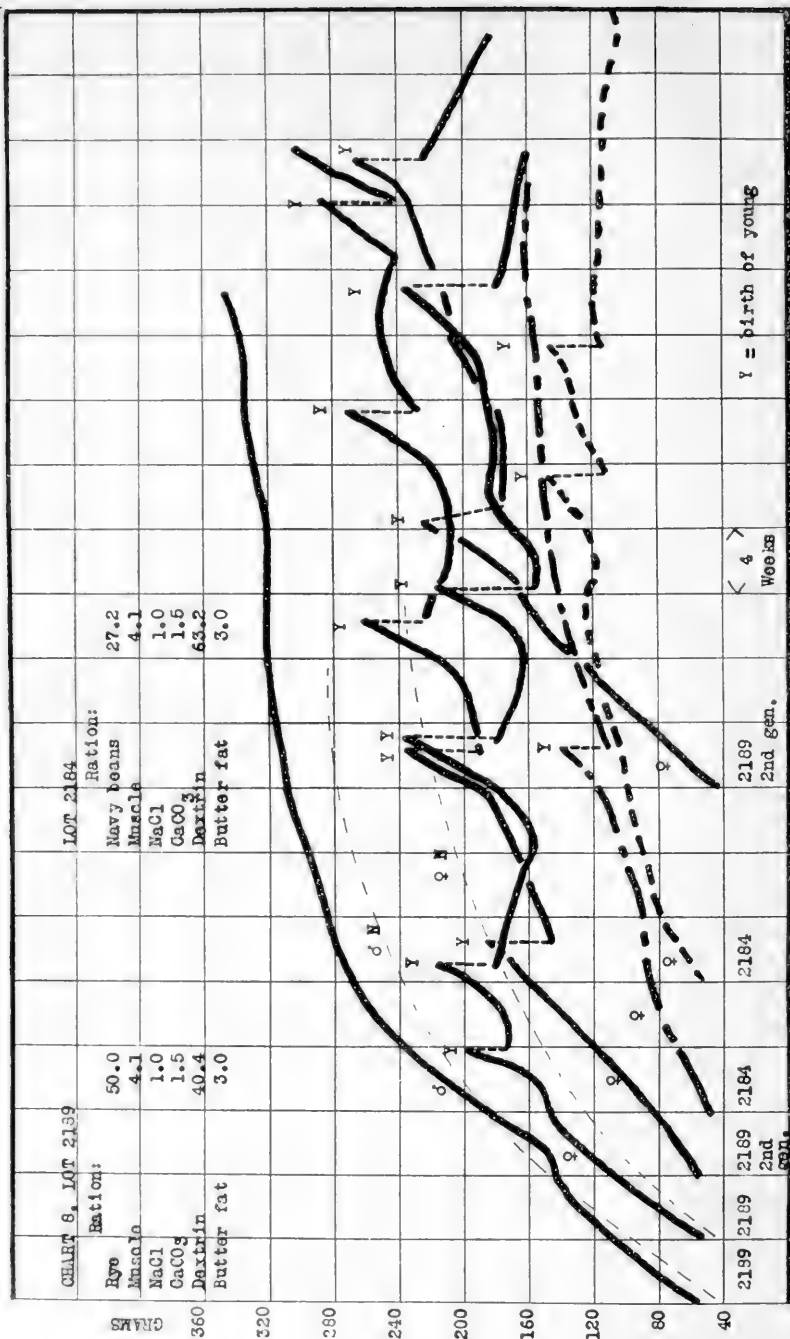


animals grew at a rapid rate to a large size. This can be interpreted only to mean that there is an excellent supplementary relation between the proteins of the wheat kernel and those of muscle tissue. This was likewise true of wheat supplemented with kidney (Lot 2193, Chart 2). The span of life was, however, somewhat short. The animals looked old at 16 to 17 months, whereas they remain vigorous up to 2 years at least when the nutrition is highly satisfactory. That they were not in this optimal condition, notwithstanding their large size and splendid fertility, is shown by the high mortality among their young. Although the biological value of the protein was high and the plane of intake sufficient to support growth and reproduction, the amount of protein of this quality was not good enough to maintain physiological well being to an advanced age or to suffice for the nutrition of the nursing young.

Two females grew up on this diet. They had collectively sixty young (eight litters) of which only twenty-nine were weaned. Two females of the second generation had sixty-five young (eleven litters) and weaned twenty-three. One third generation female had nineteen young (three litters) and weaned only three. Many of the young were destroyed shortly after birth, but those which were weaned were in good condition. In several instances the mothers reared large litters (nine young; seven young). This shows that even 9 per cent of protein of the excellent quality formed by wheat and muscle in the proportions here used sufficed for milk production even when the litters were large. We differentiate between high infant mortality due to infanticide and that due to undernutrition of the nursing young. It is most remarkable that rats on certain types of faulty diets should fail to exhibit the normal maternal instinct, which is very strong in this species.

*Chart 8.*—Lot 2189 was fed 9 per cent of protein in the form of rye and muscle. The cereal furnished two-thirds and the muscle one-third. The condition of these animals was not so good as that of Lot 2186 (Chart 7) fed a similar diet with the protein derived from wheat and muscle. They grew senile at about the same age as did Lot 2186.

Two females had collectively thirty-nine young (seven litters) and weaned fifteen. Two females of the second generation had forty-four young (eight litters) and weaned fifteen. Two third



generation females, had each a litter (eleven young) and weaned six. One third generation female remained sterile. The young of all generations always presented an inferior appearance. The nursing periods were very long and in some cases the young remained undersized. In one instance two young which survived to the 41st day were still nursing and weighed but 60 gm. together.

Lot 2184 was fed 9 per cent of protein in the form of navy beans (6 per cent) and muscle tissue (3 per cent). Other factors than protein were satisfactory. These rats grew poorly and their fertility was low. The growth on this diet was better than we have seen on 9 per cent of navy bean protein alone. They were very old looking at 15 months.

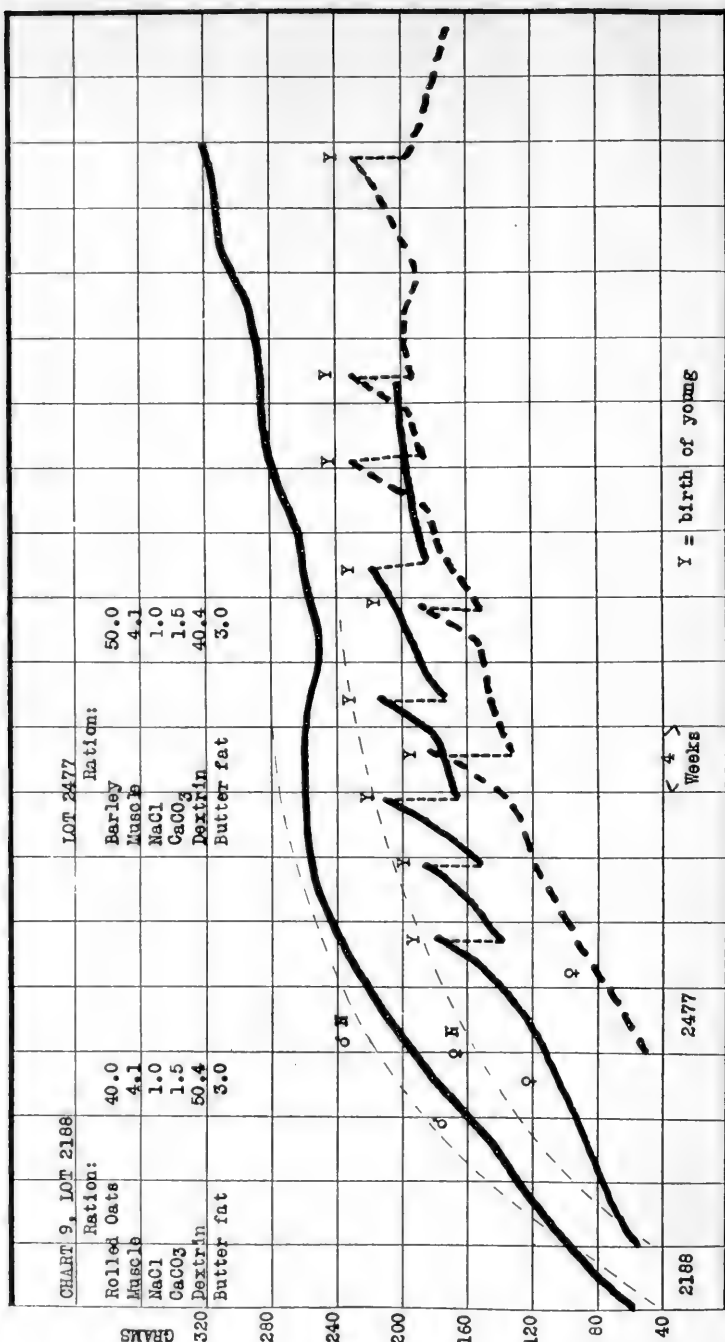
Two females had collectively ten young (three litters) and destroyed them all shortly after birth.

*Chart 9.*—Lot 2188 was fed a diet in which the total protein content was 9 per cent, two-thirds of which was furnished by rolled oats and one-third by muscle tissue. The growth curves were much better than on 9 per cent of oat protein alone (Chart 1, Lot 2484), and the animals remained longer in a good condition. They were old looking at 15 to 16 months of age. Oat and muscle proteins are not of so good quality as a similar mixture derived from wheat and muscle, but of about the same value as a mixture derived from maize and muscle. This is shown by a comparison of Lot 2188, Chart 9, with Lot 2186, Chart 7, and Lot 2187, Chart 10, both with respect to the growth curves and reproduction records.

There were two females in this group. They had seven litters (thirty-seven young) and killed them all. The young were destroyed by the mothers soon after birth. Another litter was born but destroyed before the number was ascertained.

Lot 2477 was restricted to a diet containing 9 per cent of protein. Barley furnished 6 per cent and muscle tissue 3 per cent. All other factors were made satisfactory by suitable additions. Their growth and ability to rear young were inferior to Lot 2186 (Chart 7) which was fed wheat and muscle tissue. They were very old looking at 14 months of age.

Three females in this group had collectively seventy-five young (seven litters) and weaned only two. After these two were weaned





and returned to the family cage they were killed by the older animals.

*Chart 10.*—Lot 2187 was fed 6 per cent of maize protein and 3 per cent of muscle protein. Inorganic and fat-soluble A additions were made to correct the deficiencies in the maize and muscle mixture. The growth curves of this lot were distinctly below the optimum and inferior to the group fed wheat and muscle proteins. At 16 to 17 months they were very old looking and their offspring all presented an inferior appearance.

Two females had collectively thirty-three young (six litters) and weaned thirteen. Two more litters were born but were killed by the mothers immediately after birth and the number was not ascertained. The nursing period in these animals was greatly prolonged because of the stunted condition of the young. Two females of the second generation were confined to this diet. They grew very poorly and never had any young.

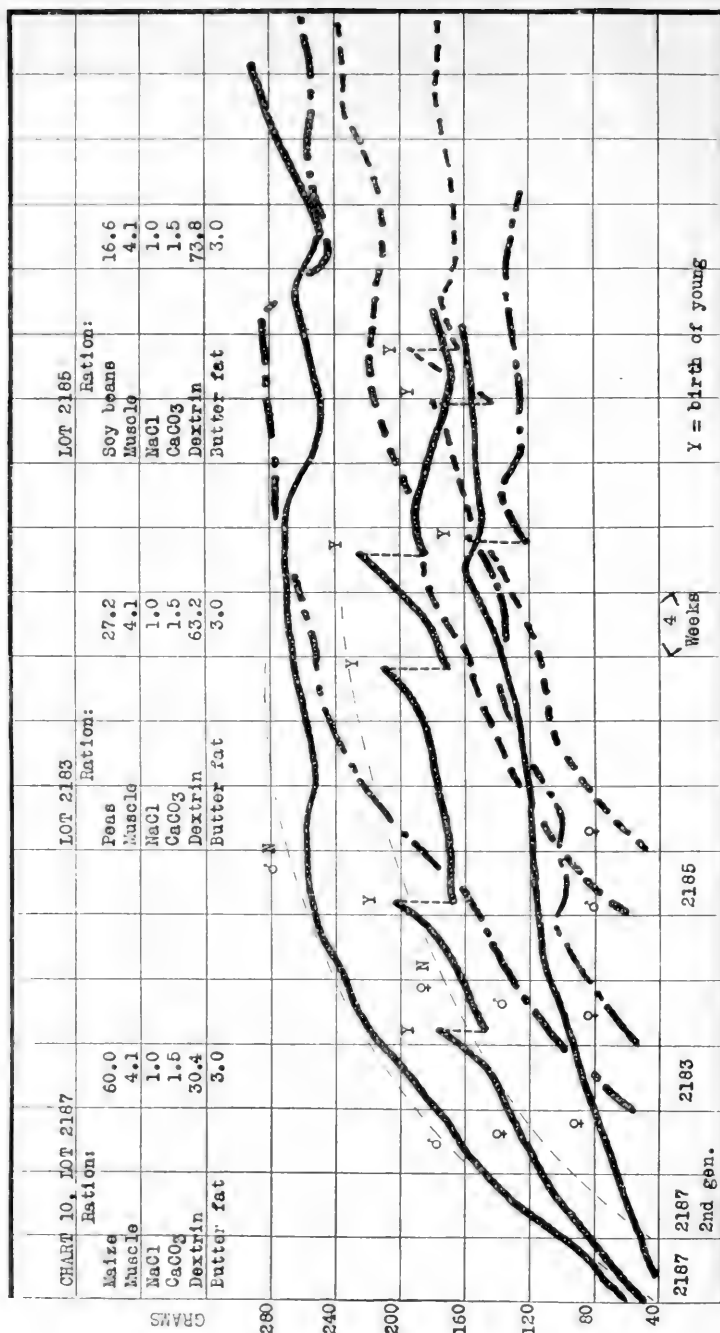
Lot 2183 was fed 9 per cent of protein derived from peas (6 per cent) and muscle (3 per cent). The growth of the rats restricted to this diet was not uniformly good, some reaching the full adult size in the usual period for well fed rats, others remaining permanently undersized. The curves of growth were far superior to any we have ever seen on 9 per cent of pea protein alone (*Chart 1, Lot 2376*). They were rough coated, old looking, and irritable at 12 months of age.

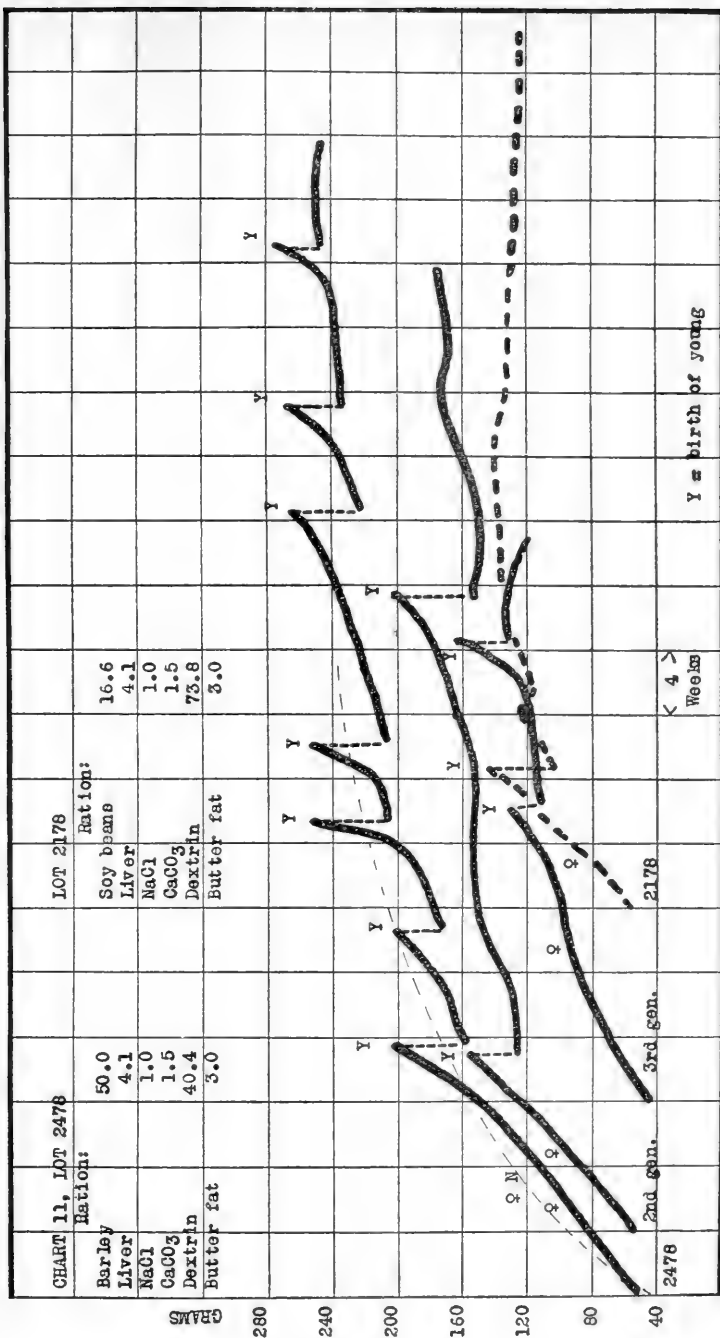
Two females had each a litter of young (seven young collectively) and destroyed them soon after birth.

Lot 2185 was fed 9 per cent of protein derived from soy beans (6 per cent) and muscle (3 per cent). The growth records were distinctly superior to those we have secured on 9 per cent of soy beans alone (*Chart 1, Lot 2510*). Their span of life was short. They were very old looking at 14 months.

There was but one female in this group. She had two litters (eight young) but destroyed them soon after they were born.

*Chart 11.*—Lot 2478 was fed a diet, the protein of which was furnished by barley and liver. It was comparable to those previously discussed in which the protein was derived from cereal or legumes, supplemented with kidney or steak, respectively. The growth and reproduction records of these animals show clearly that liver is superior to muscle as a supplement to barley (compare





Lot 2477, Chart 9 with Lot 2478, Chart 11). These animals were in good condition at 16 months of age, although their coats were beginning to be rough.

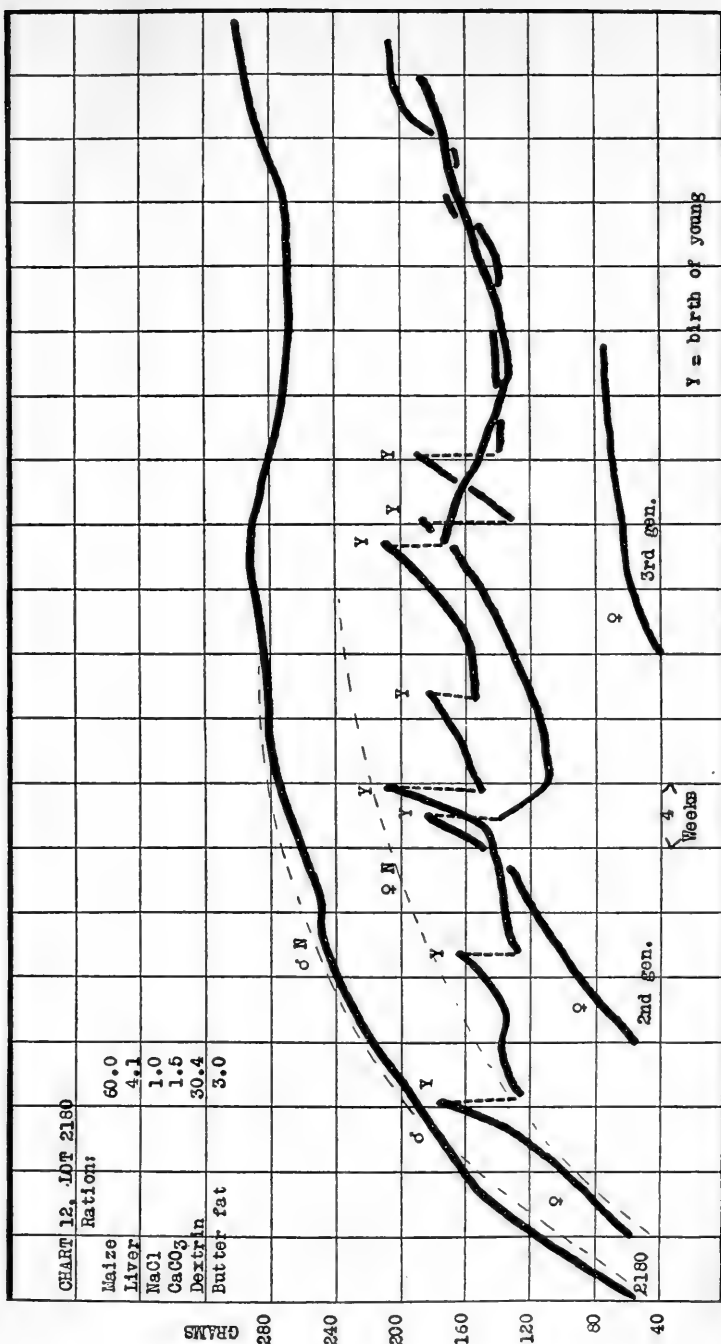
Three females were restricted to the diet. One whose growth was poor was sterile. The other two had collectively fifty-four young (ten litters) and weaned twenty-two. Four second generation females were kept on the diet. Two of these failed to grow in a normal manner and remained sterile. The other two had twenty-six young (four litters) and weaned twenty-five. One third generation female had nine young (two litters) and weaned them all.

Lot 2178 was fed 9 per cent of protein, two-thirds of which was from soy beans and one-third from liver. The growth curves were distinctly below normal, but very much better than could be secured with 9 per cent of soy bean protein alone (Lot 2510, Chart 1). These animals were undersized and old looking at 14 months, which was somewhat earlier than in the case of Lot 2478 fed barley and liver, and distinctly earlier than the time of appearance of senile characteristics in rats fed wheat and liver (Lot 2179, Chart 15).

Two females had collectively eighteen young (three litters) and destroyed them soon after birth.

*Chart 12.*—Lot 2180 was fed maize and liver proteins as 9 per cent of the food mixture. As in the other diets described in this paper the cereal furnished 6 per cent and the glandular tissue 3 per cent. These growth curves were superior to what could be secured on 9 per cent of maize protein alone, but not so good as were observed in rats fed oats and liver (Lot 2181, Chart 13) or wheat and liver (Lot 2179, Chart 15). These animals were very old looking at 16 months and the young were always undersized and inferior in appearance.

Two females had collectively fifty young (seven litters) and weaned but eight. The cause of the infant mortality in this family was mainly due to inadequate nutrition during the nursing period. This was greatly prolonged because of the stunted condition of the young. Those restricted to the diet after weaning did not thrive. Even in litters which were steadily reduced by the death of individuals at intervals of several days, the survivors did not grow better because of the lessened competition for the mother's milk supply.



Two second generation females grew up on the diet and had collectively twenty-seven young (four litters) and weaned fourteen of them. One third generation female was restricted to the diet but never grew beyond about 75 gm.

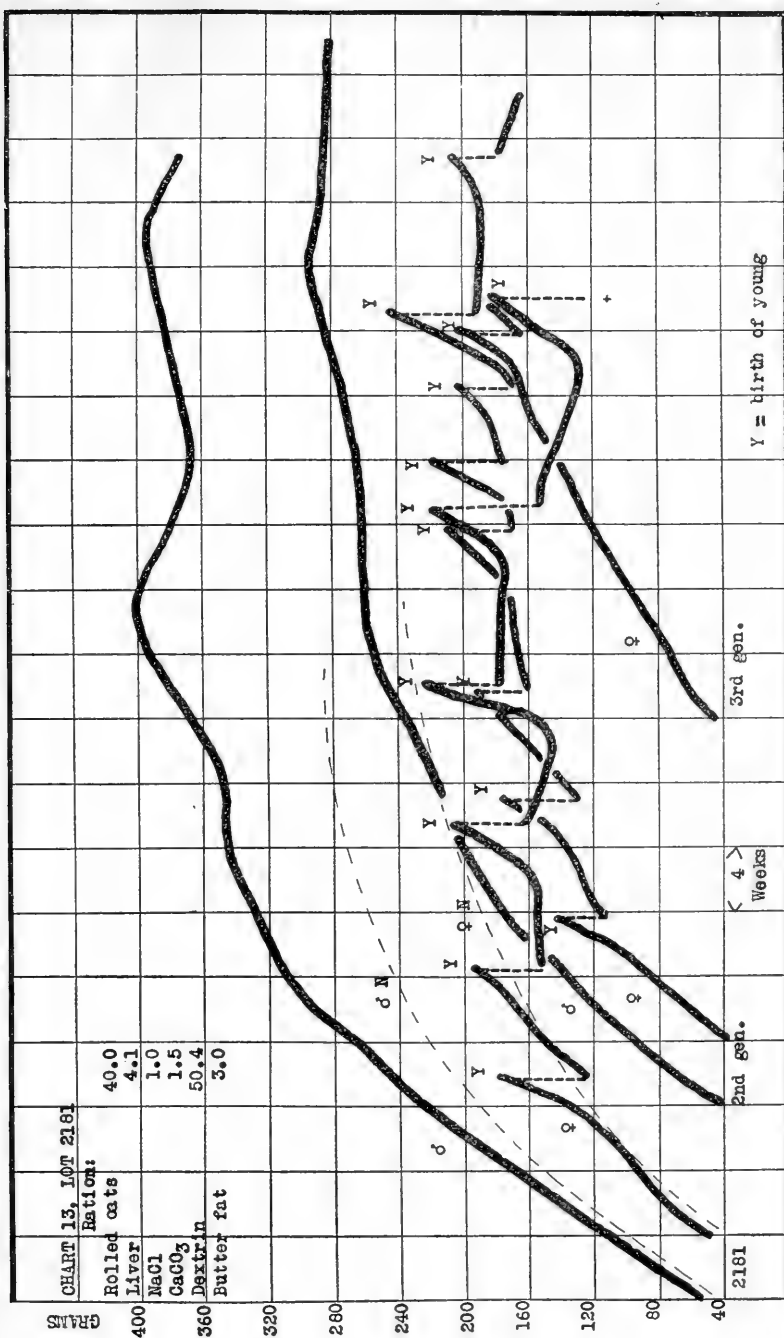
A record of infant mortality such as that of this group we know to be the result of poor quality or lack of sufficient quantity of milk secreted by the mother. It seems justifiable to conclude that it was quality rather than the amount of milk which was the determining factor, since the nursing periods were in most cases so prolonged beyond the normal period of 25 days. If the milk flow had been low it would hardly have persisted over so long a period and have enabled a few young to at last reach a state of independence. The young in the second generation were distinctly better once they became able to eat the diet on which the mother was producing milk, than they were while still so immature as to be confined to their mothers' milk as their sole food.

*Chart 13.*—Lot 2181 was restricted to a diet of rolled oats and liver, the plane of protein intake being 9 per cent of the food mixture. The oats supplied 6 per cent and the liver 3 per cent of the total protein. The inorganic and fat-soluble A deficiencies were made good by suitable additions of salts and butter fat. The growth curves of the group were normal and fertility essentially so, but the mortality of the young was high. The rats in this group began to look old at 14 months and deteriorated rapidly thereafter. The growth curves of this group were far superior to those of Lot 2188 (Chart 9) which grew up on a similar diet with beef muscle in place of liver.

Two females in this group had collectively sixty-eight young (ten litters) and weaned forty. Two second generation females had forty-three young (ten litters) and weaned fourteen. One third generation female had a litter of seven when she had been 6 months on the diet after weaning. She weaned six of these.

All the animals of this group were killed at about 18 months of age, and while they presented shabby exteriors their organs were found at autopsy to be in good condition and there were no lung infections visible.

*Chart 14.*—Lot 2182 was fed 9 per cent of protein, 6 per cent being furnished by rye and 3 per cent by liver. The growth curves were excellent and the fertility was high. This shows that this







combination of rye and liver proteins is of excellent quality, otherwise 9 per cent of protein would not have sufficed to promote growth so satisfactorily. The infant mortality of this group was, however, very high. This was due to the inadequacy of this amount of protein, even of excellent quality for growth, for the elaboration of milk of suitable quality for the nutrition of the young.

Two females had collectively sixty-one young (nine litters) and weaned thirty-four of them. Three second generation females had fifty young (ten litters) and weaned only eight of them. One of these three had a litter of three and thereafter went 12½ months before having a second litter. One rat (a male) of the third generation was restricted to the diet and grew to a size nearly normal to the adult.

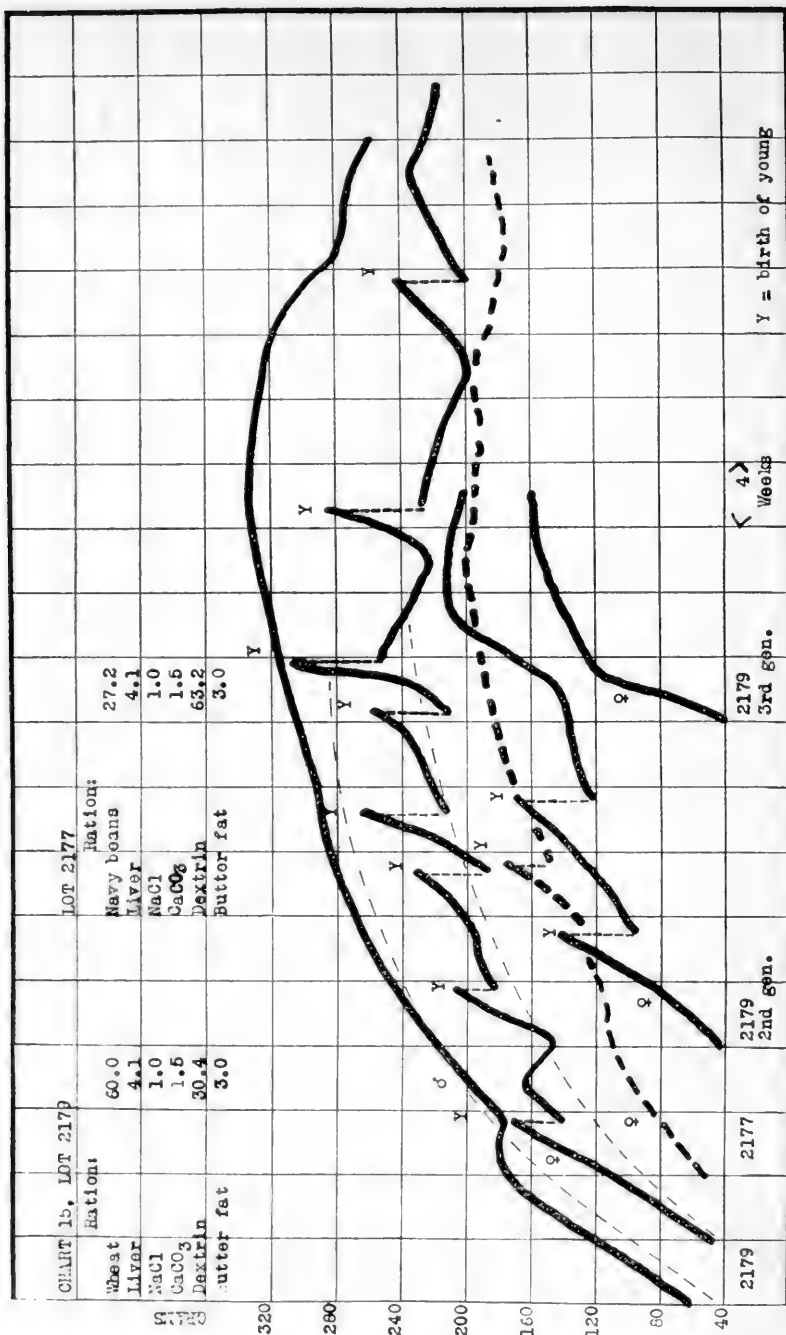
The rats in this group were first observed to show the coat changes characteristic of incipient retrogression toward senility at about 15 to 16 months.

Lot 2176 was fed a 9 per cent diet derived from peas (6 per cent) and liver (3 per cent). The growth curves were poor as compared with any secured with combinations of cereals and liver in comparable amounts. They were undersized and their fertility was low. They assumed the characteristic coat which we have taken as an index to the transition from the youthful to the senile condition at about 13 months.

Three females were restricted to this diet. One had a litter of five young but destroyed them soon after birth. The other two remained sterile.

*Chart 15.*—Lot 2179 was fed 9 per cent of protein, 6 per cent being derived from wheat and 3 per cent from liver. Their growth curves were normal but they were never in so good a condition as those fed either oats or rye with liver (Lot 2181, Chart 13 and Lot 2182, Chart 14). Their fertility was very high but the mortality of the young was great. They began to look old at 18 months of age.

Two females had collectively 127 young (eighteen litters) and weaned forty-two. Most of these young were destroyed by the mothers soon after birth. The mothers were apparently in good condition and the destruction of the young can be accounted for only on the basis of a peculiar psychological reaction of the mother owing to the nature of the faulty diet.



Three second generation females had collectively twenty-three young (four litters) and weaned only one. This sole survivor was kept on the diet for  $3\frac{1}{2}$  months but was inferior in development and was discarded.

Diets of this type in which the protein intake was about 18 per cent, and in which 11 per cent was furnished by cereals and legume seeds and 7 per cent by liver, kidney, or muscle, have proved excellent for promoting fertility and rearing of young with very low mortality. This proves conclusively that in the series of experiments reported in this paper the limiting factor in growth, fertility, and success in rearing young was protein.

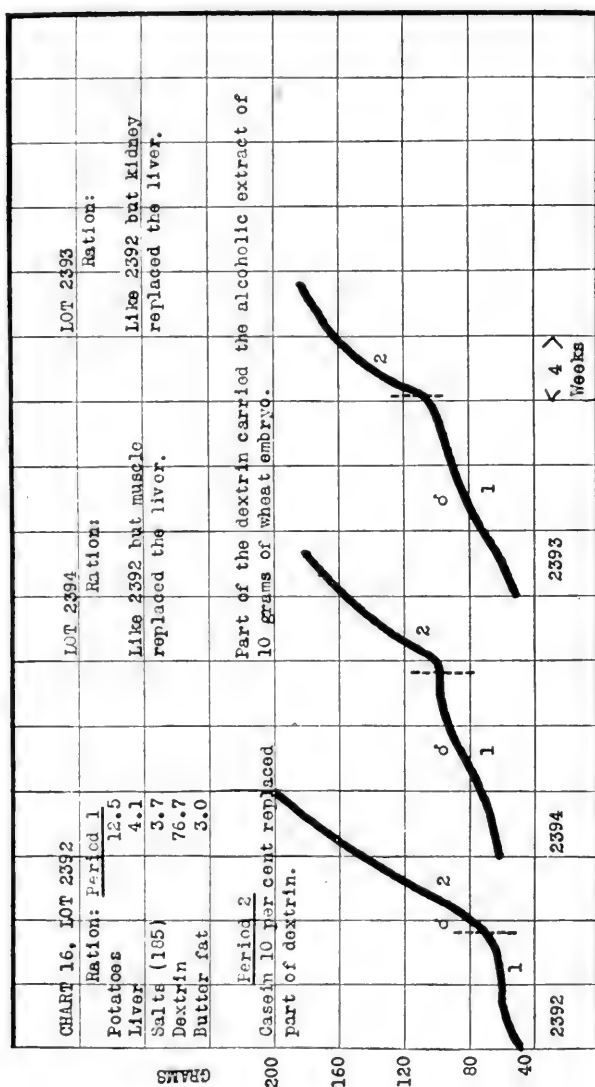
Lot 2177 was fed navy beans to furnish 6 per cent of protein and liver to furnish 3 per cent. The growth curves were inferior to any observed on combinations of a cereal and liver, but comparable to those of rats fed peas and liver in comparable amounts (Lot 2176, Chart 14). Growth was, however, far superior to any secured with 9 per cent of navy bean protein alone (Lot 2368, Chart 1).

These animals appeared *very old* at 15 months. There were three females in the group. One had a litter of two which she destroyed soon after birth. The other two remained sterile.

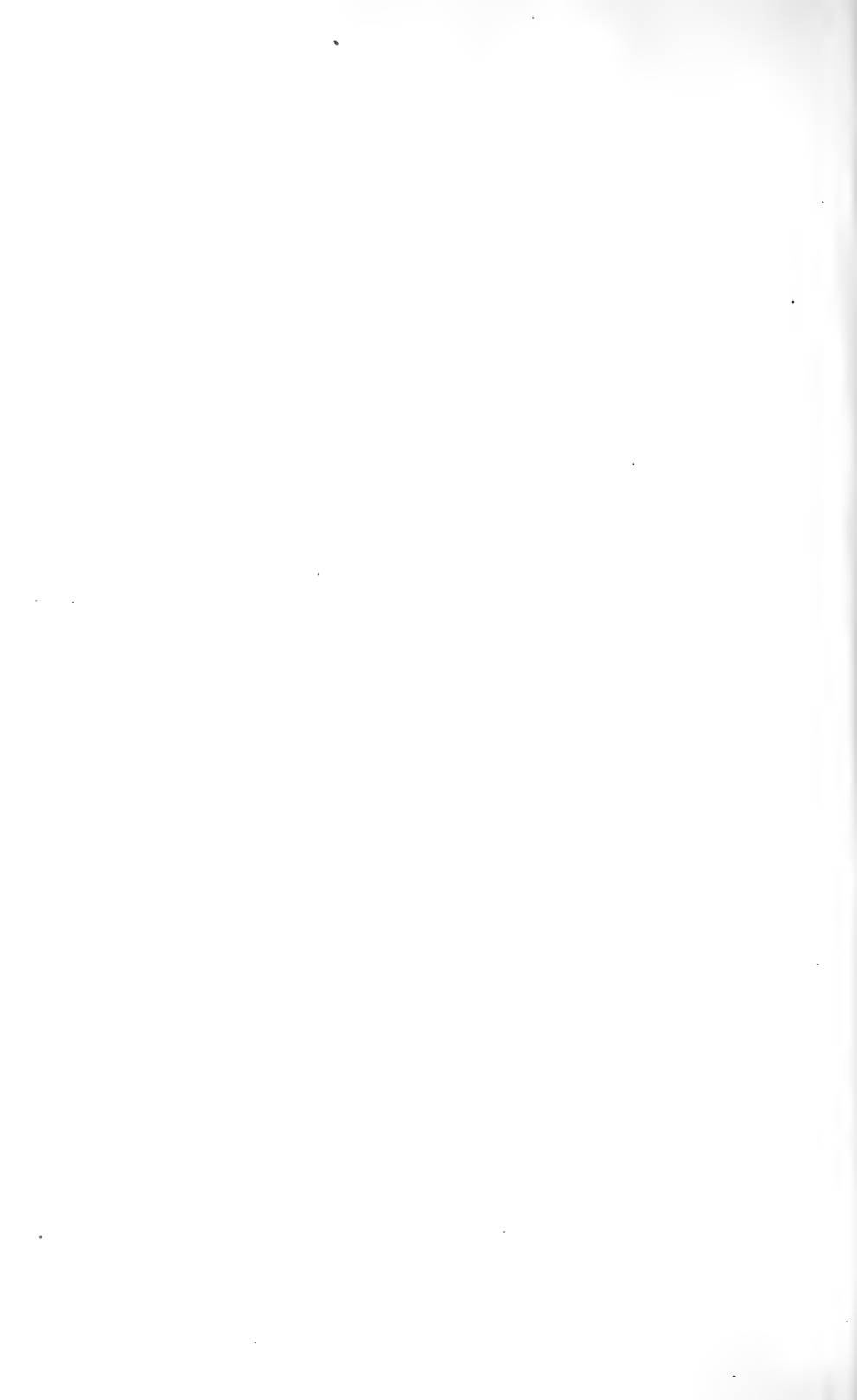
*Chart 16.*—Lot 2392 was fed a diet containing but 4 per cent of protein derived from potato (1 per cent) and liver (3 per cent). A very small amount of nitrogen in this and the following rations was added in the alcoholic extract of wheat germ. The rats were unable to grow on this diet, all factors in which, other than protein, were of fairly satisfactory quality. In Period 2 purified protein (casein) was added. This modification of the diet was followed by a sharp response to growth.

Lot 2394 was fed a diet identical with that of Lot 2392 except that muscle tissue (round steak) replaced the liver. The animals grew very slowly on this food mixture containing but 4 per cent of protein. In Period 2 the protein moiety of the diet was enhanced by the addition of casein. This led to growth at a rapid rate.

Lot 2393 was fed a diet identical with those of Lots 2392 and 2394 except that kidney replaced the liver and muscle, respectively. This diet also contained 4 per cent of protein. Growth at a slow rate was possible on this diet. In Period 2 casein was added and this led to a stimulation of growth. It will be seen from the curves



presented in the chart, each of which is representative of a group of four animals, that kidney protein supplements the nitrogen of the potato better than either liver or muscle proteins. These curves are included in this series to illustrate the fact that certain combinations of proteins are of sufficiently good quality to enable young rats to grow nearly as well on diets containing but 4 per cent of protein (potato and kidney) as they do on rations of similar value with respect to non-protein factors in which the sole source of nitrogen is 9 per cent of legume proteins (pea, navy bean, and soy bean). Compare Chart 1, Lots 2376, 2368, and 2510 with Chart 16.



## SUPPLEMENTARY PROTEIN VALUES IN FOODS.

### III. THE SUPPLEMENTARY DIETARY RELATIONS BETWEEN THE PROTEINS OF THE CEREAL GRAINS AND THE POTATO.

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(Received for publication, March 21, 1921.)

It has become well established that the diet of man or animals may, when defective in sufficient degree, produce at least two distinct syndromes, scurvy and beri-beri. Experimental studies on animals leave no room for doubt that a third specific deficiency disease, xerophthalmia or keratomalacia, is produced by lack of a definite organic factor essential for normal nutrition. There is much reason to suspect that certain eye conditions popularly referred to as night-blindness, and in the literature as hemeralopia may be, in part at least, due to lack of sufficient fat-soluble A. The widespread infections of the eyes seen in Egypt and elsewhere, may have their origin in lowered vitality of the eye structures due to lack of the dietary factor, fat-soluble A, which favors the invasion of the eyes by microorganisms. The evidence seems sufficient to warrant the conclusion that there are three deficiency diseases which occur in man as well as in animals.

It is natural that the first step toward an appreciation of the relation of the diet to health in man should have been the result of casual observation on the extreme conditions, scurvy and beri-beri. The rarity of reference to the relation of faulty diet to the general health of peoples in the writings of those who are now discussing health problems, shows that it is not at present accepted by many that there is any important relation of the food supply to well being, except in extreme cases of one-sided diet in which the defects are sufficiently grave to induce the development of a "deficiency" disease. The reason for this is easy to understand. Our standards of what constitutes wellness and illness, as well as of what con-

stitutes normal physical development, are relatively low. People are generally considered well until they admit that they are ill, or until they look so unwell as to attract attention. The difference between the average physical development of men and women, as contrasted with the best specimens in the community, is very great, yet we are accustomed to regard as normal many persons who are but poorly constituted physically.

Malnutrition is painless, and leads to susceptibility to other agencies which may induce discomfort. The sequence of events is not ordinarily appreciated, and the cause, therefore, escapes notice. Low vitality, low resistance, and inefficiency, and a tendency to cumulative fatigue, are what we should expect in man to result from adherence to a faulty diet when the faults are of a lower order than would be necessary to bring about an attack of a "deficiency" disease. Although a few leaders in the new movement for the betterment of the health condition of children are urging the importance of properly selected food, the idea is new and not generally appreciated. There is much reason to believe that one of the most important contributing factors in industrial fatigue is the low recuperative power that results from an unsatisfactorily selected food supply. The data regarding the animals described in this paper, all of which were subjected to diets which were faulty to a certain extent, afford striking evidence that such types of diets interfere with the completion of the life history in a normal manner. This is true, notwithstanding the fact that in some instances the faults were by no means marked. The extent to which the animals were injured, and the manner in which they were affected, afford food for serious reflection concerning the similar experience of man and its probable causes.

The results of our experimental studies on the rat have led us to an appreciation of the short-sightedness of the view that a diet is satisfactory if it is not sufficiently poor to cause the development of a "deficiency" disease. Although the importance of a diet containing an abundance of milk and eggs has become generally accepted to have a distinct therapeutic value in the treatment of tuberculosis, it has not been widely appreciated that a similar diet should be very effective as a preventive of infection or of the flaring up of the disease.



We have given a great deal of attention to the problem of demonstrating the effects of relatively slight defects in the diet on the general health of the rat, and on its capacity to reproduce and rear young, and to remain vigorous to an advanced age. We have united the study of these problems with the study of the extent to which the proteins of certain foods supplement those of others. It has been easily possible to develop these lines together, and indeed it was not possible to pursue the one phase of the work independently of the other, for in such systematic studies one meets with the nice gradations in quality of proteins which afford the opportunity to make refined observations on the physiological well being of the animals as affected by the variations in the diet.

We deem it a matter of great importance to discover in its incipency when an animal is brought into a state of nutritive instability. Under such conditions it is unable to support a burden of any appreciable magnitude, and it matters little whether the burden be that of producing milk, or bearing young, or of withstanding fatigue. It is such shades of difference in quality which occur in human experience in numerous cases. The type of experiment which we have come to employ, in which the performance of reproduction and nursing of young comes under observation, brings to light this "twilight zone" of nutrition in which the vital powers of the body are below the optimum.

It is desirable at this time to discuss the changes in appearance of the rat, and in its behavior as it grows old, in order to make possible an appreciation of the manner in which we have managed our experiments.

The well fed rat exhibits up to about 18 months of age the appearance of full vigor as shown by plumpness of the muscles; bright, prominent eyes; clean, smooth, pink skin, easily observable on the feet and ears. The tail appears clean and free from scales. The coat appears to consist of hairs of approximately uniform length, and is glossy and well kept. Such animals are not apprehensive and permit themselves to be handled without becoming annoyed. They do not attempt to escape when handled with care.

At a certain point in their life history there is a characteristic change in the appearance of the coat. This consists of the prominence of certain hairs which are scattered over the entire body, and which look coarse and are distinctly longer than the under coat.

Physiologically youthful rats, irrespective of age, give attention to their coats. When the latter differentiates into hairs of two sorts the animals become neglectful of their toilets and look less clean and attractive than formerly. The skin on the ears, feet, and tail generally loses its waxy and healthy surface and appears dry, harsh, and often scaly.

With this change in appearance there is usually seen a change in the habits of the animals. They tend to sit and sleep with the head tucked under the body or with the face downward. Healthy youthful rats sleep lying stretched out on their ventral surface or partly turned on one side, with the front legs extended and with the head resting on the paws. When the cage of youthful rats is opened they are awakened if asleep, and show an interest in their observer, but do not show signs of fear. Animals which are developing senile characters are generally dozing and their attention is not usually attracted by careful opening of the cage. When touched they spring up in surprise and show irritation at being molested. As time passes this behavior becomes more marked. The irritability, somnolence, dirtiness, roughness of the coat, and scantiness of hair become progressively more noticeable. To some extent, according to the nature of the fault in the diet, specific changes appear, such as attenuation of form and baldness over limited areas, but these latter are more characteristic of animals fed certain foods than they are of diets from different sources having like specific dietary deficiencies. A further example of this peculiar effect of diet due to some obscure causes is seen in the frequent occurrence of very fine, short, glossy hair, suggestive of a mole in rats fed largely on maize or kafir corn.<sup>1</sup>

Another type of observation which has proved to be of value as an index to the quality of the diet which enables one to make fine shades of distinction in the value of the protein factor in a series of experiments which are properly planned, is the success of the females in rearing their young. This is a more sensitive index than is fertility, although in general low fertility and high infant mortality tend to run parallel. The fertility may, however, be high, and but a small number of young be reared to the weaning age. This mortality is, we believe, generally due to poor quality

<sup>1</sup> McCollum, E. V., and Simmonds, N., Unpublished data.

of the milk secreted by the mothers rather than lack of milk production. It is not always possible to get satisfactory information on this point, but since the stomach when filled with milk can be readily seen in a young rat during the first few days when the animal is properly illuminated, we have been able to observe the amount of milk produced in a sufficient number of cases to lead us to believe that in general the failure of the young to grow is due to deficiencies in the composition of the milk rather than to lack of sufficient amount.

There is a marked tendency among female rats fed such diets as are described in this paper to destroy their new-born young. If for any reason they become scattered about the cage the mothers will not be solicitous about returning them. This abnormal psychological reaction of the mothers toward their young represents a new and hitherto unstudied effect of faulty nutrition. It serves to emphasize the deep seated relation of the character of the diet to the mental reactions generally, for if the maternal instinct is so perverted, those attributes of the nervous system which are less deeply ingrained should be influenced in even greater degree.

In contrast to the behavior of the mothers on experimental diets, details of which are given in the legends to the charts, it is of interest to contrast the behavior of the mothers in our breeding stock. It is our custom to isolate the females when it is easily observable that they are pregnant. This cannot be determined with certainty until about the end of the second week after fertilization. For some years it has been our custom to isolate two or three females in our breeding stock in a single cage. They each prepare a nest for the reception of their young, but when the cage is cleaned within a few days, and fresh bedding replaces the old, the young are mixed and returned to the cage together. From this time on the mothers do not separate their own young, but allow them to remain together in one nest, which may contain as many as twenty to thirty individuals. They nurse each other's young indiscriminately. A mother will spread herself over the nurslings, and as many as possible attach themselves to her. After a time she will free herself from them and another mother will take her place. They seldom kill a young one and the infant mortality is almost zero. Only very infrequently have we seen a young rat under such circumstances unable to compete successfully with the others for his share of the milk supply.

In experimental animals on diets which are faulty, it appears, as stated above, that the milk flow is ordinarily sufficient to meet the needs of the developing young, but the quality may be poor with respect to some one or more factors, and cause failure of the animals to grow satisfactorily. It appears, however, that some mothers do not produce enough milk, at least after a time. We have occasionally seen a litter grow fairly well for 4 to 10 days, then all die within a few days. The cause of such behavior has in general not been ascertained.

We have given some attention to determining how long a female rat may continue to give milk provided she is kept with young of a suitable size. In one case in which the young were removed and replaced by smaller ones at intervals of a few days, a female rat continued to secrete milk up to 141 days in quantity sufficient to induce some growth in four young about 8 to 10 days old. It is known that these young did not eat of the mother's food supply.<sup>1</sup>

When the fault or faults in a diet are sufficiently marked, growth is interfered with in the young, and the effects soon become apparent in the external appearance of the animals, even in the adult. We have made an effort to determine *how sensitive* the rat is to slight degrees of faulty composition in its diet. This can be done by prolonging the period of observation far beyond the growth period, and including a record of the fertility and success in rearing young and the time of the appearance of the first signs of old age. Since the character of the diet is but one of the factors which influence the well being of an animal, we have maintained the hygienic conditions such as illumination, temperature, ventilation, and opportunity for exercise uniform throughout our entire rat colony. Furthermore, the differences in the condition and vitality of the animals as measured by the tests described are due in all cases to variation in the quality of the diet, which falls within the limits of what may occur in combinations of ordinary natural foods of animal and vegetable origin.

As an example of the objective which we have had in mind in conducting these studies, the comparison of the relative values of the proteins in the several rations may be cited. The value of the proteins in a food or mixture, or the value of a purified protein, has not been studied by methods more sensitive than that of comparing their efficiency for the support of growth. We have learned

that much more accurate comparison of quality in proteins can be made by maintaining a series of animals on a diet otherwise comparable, but containing various levels of the same source of protein throughout the growing period and into adult life, so as to discover the plane which just suffices for this result without inducing inferiority in appearance or size. Under these conditions the mother rat should she produce and attempt to suckle a litter would be confronted with an added demand for nutriment by the young. The young at the nursing age are more sensitive to faulty diets than are older animals which have been satisfactorily nourished during the first weeks of life. We have thus set up experimental conditions in which an unusual demand is made upon the mother, and extraordinary sensitiveness is characteristic of the young because of their early age and lack of reserve power.

It is of more than ordinary interest to find, as we have, that a diet may be sufficiently good to enable a group of animals to grow to apparently the normal adult size, and even to support a fair amount of reproduction and moderate success in the nursing of young to a state of independence, and yet be inadequate for the nourishment of a family through several generations. This fact is brought out well in many of our studies in which families during several generations have been restricted to a monotonous diet. If the diet is properly constituted with respect to all its parts the animals tolerate, at least to the fifth generation, such a monotonous food supply without any signs of injury. If, however, there are factors which are even slightly below the optimum, the vitality of the animals falls off in each succeeding generation, and the strain dies out. Sometimes this happens in the second, third, or fourth generation.

An interesting difference in the effect of stunting of young animals is seen, depending on the cause of the interference with growth. When the protein content of the ration is too low, young rats remain approximately normal in form, or at least they remain long and lithe. When the inorganic content of the ration is faulty on the other hand, they develop a short and stocky form. In the former case they respond to a better protein supply and look approximately normal, but when deformed by lack of sufficient calcium they never assume the normal form as the result of correction of the diet.<sup>1</sup>

The discussion of the growth, fertility, infant mortality, and appearance of the first recognizable signs of senility which is presented in the legends to the charts, will make clear the fact that even very slight differences in the quality of the food mixture with respect to protein and fat-soluble A at least, can exercise a marked influence on the vitality of the rat. Animals may be brought to a state by feeding ordinary cereals and potato, supplemented in a fairly satisfactory manner except for the protein factor, in which their nutritive condition is unstable, and where very slight shifts in the character of the food for better or worse, make easily observable differences in their capacity to perform satisfactorily the functions of reproduction, or to retain full vigor to an advanced age.

The experimental work reported in this paper is limited to a study of diets in which the protein was derived from a mixture of a cereal grain and potato. The quality of protein was estimated as accurately as possible by existing methods. In some cases casein was added to the diet in order to bring out the fact that the protein in a certain mixture was actually the limiting factor. The results show that the potato nitrogen serves to some extent to supplement that of the cereal grains, but that it is not so valuable for this purpose as are certain animal tissues such as kidney, liver, or muscle.<sup>2</sup>

The data furnish definite evidence that there is no marked difference in the capacity of the nitrogen compounds of the potato to enhance those of the different cereal grains, as was found to be the case in similar combinations of cereals with animal tissues. The latter are individually more effective with certain grains than with others, whereas the potato is less highly valuable in any combination, and more similar in its relations to each.

These studies lend support to the general proposition that it is not wise to attempt to combine foods which have the same function, such as have the seeds and tubers. Both are storage tissues of plants and have in great measure the same dietary properties. They do not, therefore, serve effectively to make good each other's shortcomings.

<sup>2</sup> McCollum, E. V., Simmonds, N., and Parsons, H. T., *J. Biol. Chem.*, 1921, xlvii, 139.

## SUMMARY.

We have described methods of experimenting which are more sensitive than rate of growth for yielding information concerning slight gradations of quality in foods. These involve fertility, success with the rearing of young, longevity, the preservation of youthful characters, and the stability of the nervous system. By establishing standards for comparison these indexes to well being reveal with great refinement the physiological condition of the animal.

The supplementary values of the nitrogen compounds of the potato for those of the cereal grains and legume seeds have been studied with a view to making refined observations as to the biological values of several combinations. These experiments have brought to light the following facts:

The nitrogenous compounds of the potato tend in some degree to enhance the biological value of proteins of cereals and legume seeds, but not to as great an extent as do the proteins of such animal products as kidney, liver, muscle,<sup>2</sup> or milk.

Marked differences are observed in the extent to which animal tissues enhance the quality of the proteins of individual seeds. No such selective efficiency has been observed in the relation of the amino-acids of the potato to those of cereal or legume seeds. The potato seems about equally effective as a supplement for all which have been studied.

*Chart 1.*—These records are typical growth curves of rats fed diets in which wheat, rolled oats, maize, peas, and millet seed, respectively, furnish the only source of protein. The amount of each seed was adjusted so as to make the protein content of the diets 9 per cent of the food mixture. In each case the necessary inorganic salts and butter fat were added to make good the well established deficiencies of these seeds.

Wheat proteins are seen to produce the best growth curves and the highest fertility. No young were secured from the animals fed maize, peas, or millet seed. A few young were reared on the wheat and oat diets. Charts 5 and 8 show that the infant mortality is in a great measure due to inadequate protein moiety of these diets. These records serve as the basis of comparison with those contained in the other charts.

CHART 1. LOT 2481

CHART 1, LOT 2481		LOT 2484		LOT 619		LOT 2376		LOT 1383	
Ration:		Ration:		Ration:		Ration:		Ration:	
Wheat	90.0	Rolled oats	60.0	Maize	90.0	Period 1	40.8	Period 1	75.0
NaCl	1.0	NaCl	1.0	NaCl	1.0	Peas	1.0	NaCl	1.0
CaCO <sub>3</sub>	1.5	CaCO <sub>3</sub>	1.5	CaCO <sub>3</sub>	1.5	CaCO <sub>3</sub>	1.5	CaCO <sub>3</sub>	1.5
Dextrin	4.5	Dextrin	34.5	Dextrin	4.5	Dextrin	53.7	Dextrin	22.5
Butter fat	3.0	Butter fat	3.0	Butter fat	3.0	Butter fat	3.0	Period 2	
						Period 2		Casein 12 per cent	
						Casein 10 per cent		replaced part of	
						replaced part of		dextrin.	

Y = Birth of young

Weeks

Lot 2481

Lot 2484

619

2376

1383



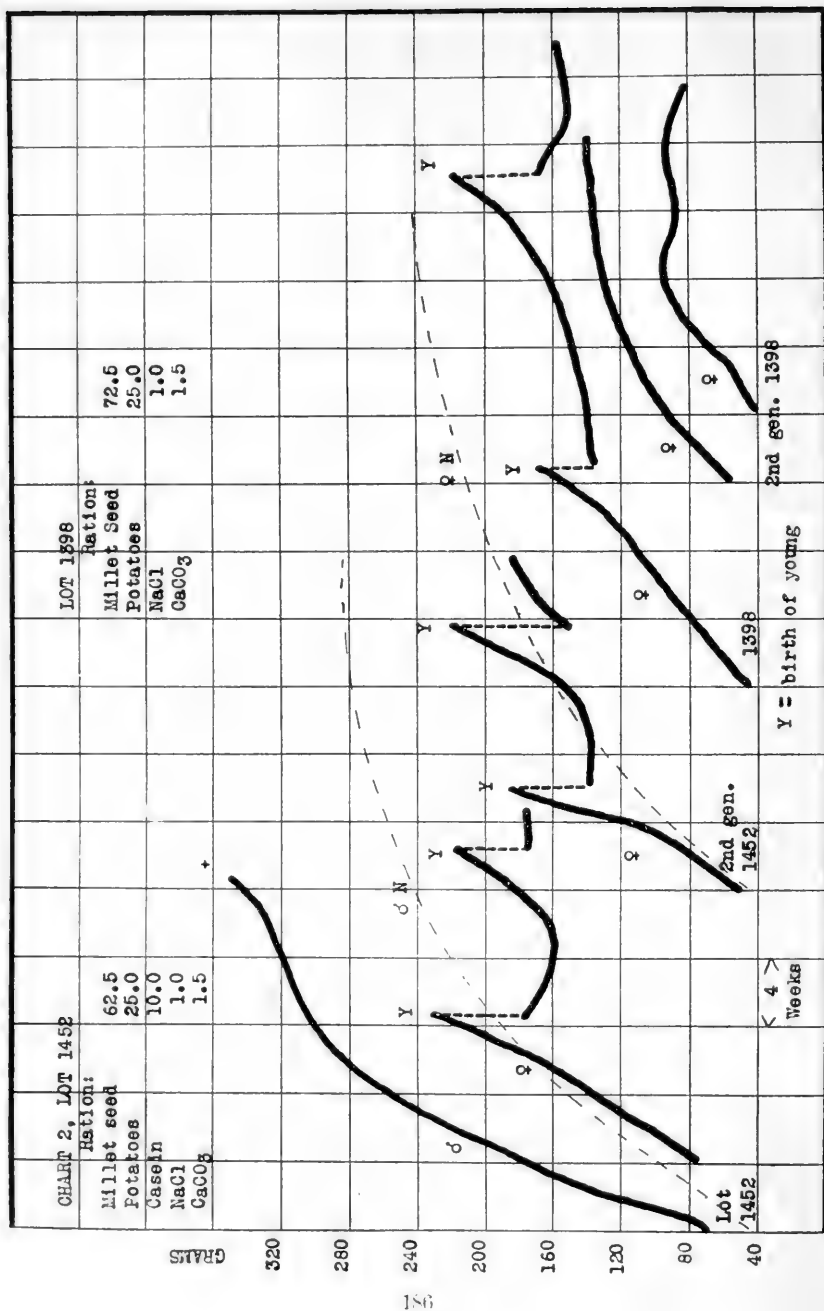
In all cases the food mixture was ground together so that all its ingredients were consumed in the proportions shown in the formula, as is our invariable practice.

*Chart 2.*—These curves illustrate the growth and reproduction of a group of rats fed from weaning time on diets, the protein of which was derived from millet seed and potato. This seed and tuber served as the only source of fat-soluble A.

Lot 1398 contained about 11 per cent of protein ( $N \times 6.25$ ), approximately 9 per cent being derived from millet seed and 2 per cent from potato. The growth records were poor, but distinctly better than was observed in Lot 1383 (Chart 1), in which 9 per cent of protein derived entirely from millet seed was the sole source of nitrogen. No young were secured in the latter group, whereas a few were secured from Lot 1398. This group contained two females. These had together eighteen young (three litters) of which nine were weaned. Two of their daughters were reared on the diet but remained sterile. The young of this group were all undersized for their age and the nursing period had to be prolonged to approximately twice the normal period of 25 days in order to enable the young to survive on the family diet. There is every reason to believe that the high mortality of the young in this group was the result of qualitative inadequacy of the milk secreted by the mothers. All these rats aged early. They were very senile at 8 to 10 months.

Lot 1452 had a diet like that of Lot 1398 except that it contained 10 per cent of casein to supplement the protein. The good effects of this addition are easily seen in the character of the growth curves. Millet seed does not appear to be a very wholesome food grain. The fertility of the females in this group was far below normal and the mortality of the young high. These young were not destroyed soon after birth but only after they had time to show from retarded growth that the quality of the milk of the mother was inferior.

Two females had together twenty-six young (three litters) of which twenty were weaned. Two daughters were confined to this diet. One had one litter of four young which she destroyed. The other had one litter which she destroyed, and died in the delivery of a second litter.



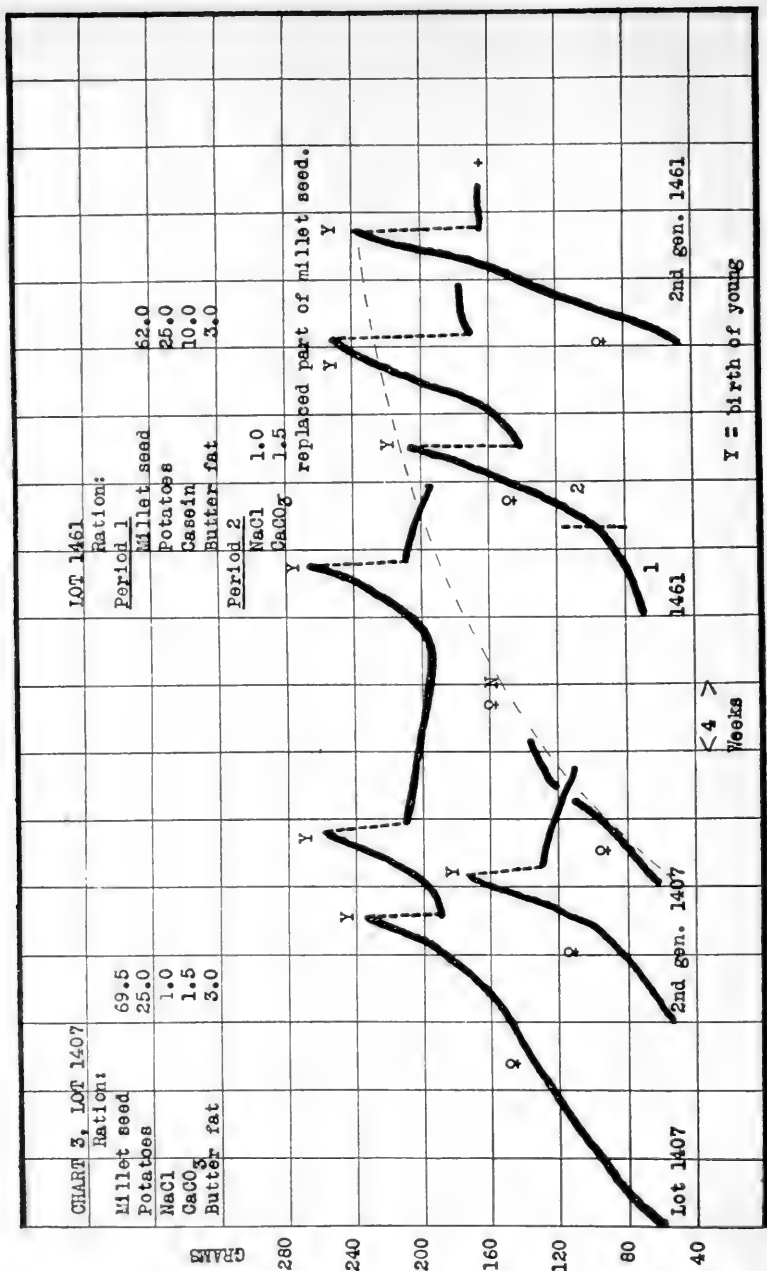
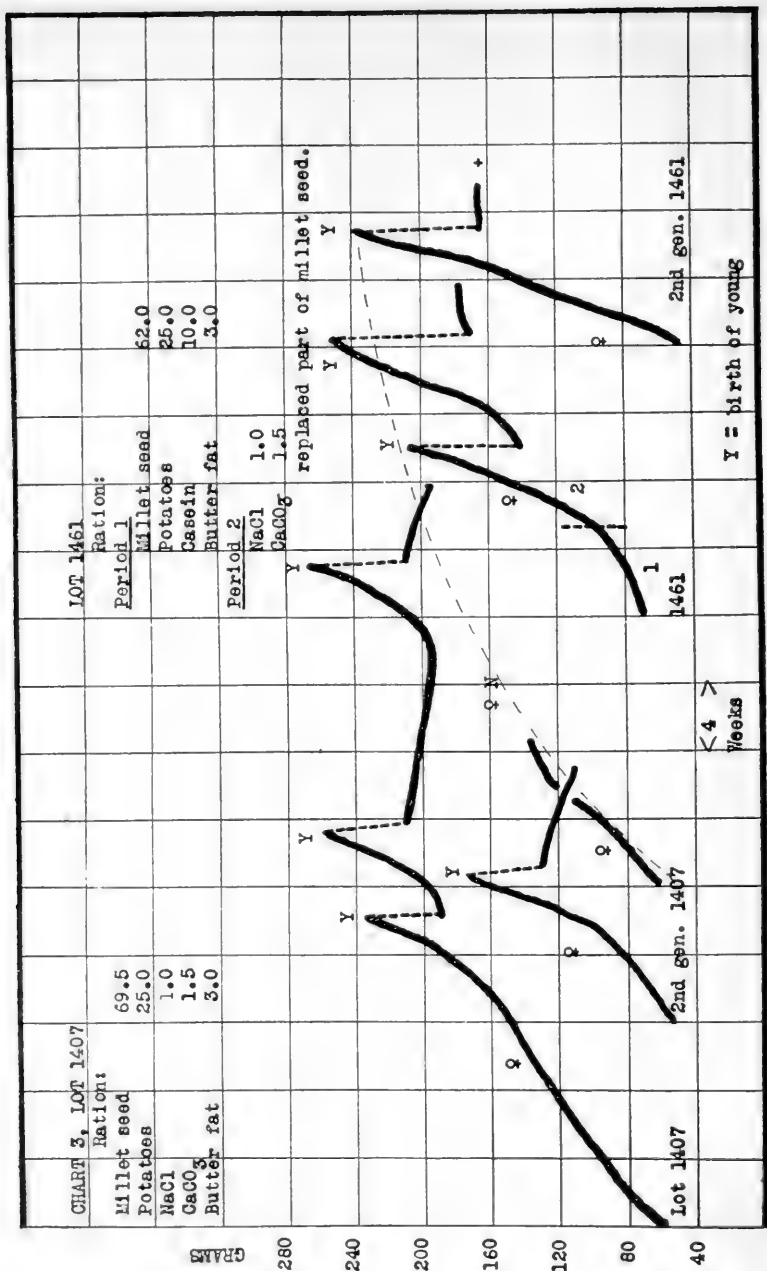
*Chart 3.*—The animals in Lots 1407 and 1461 were fed diets similar to those of Lots 1452 and 1398, respectively (Chart 2), except that the former had an added source of fat-soluble A. Since millet seed contains distinctly more of this factor than wheat or oats, or than peas or beans, the effects of adding butter fat to this ration was scarcely noticeable.

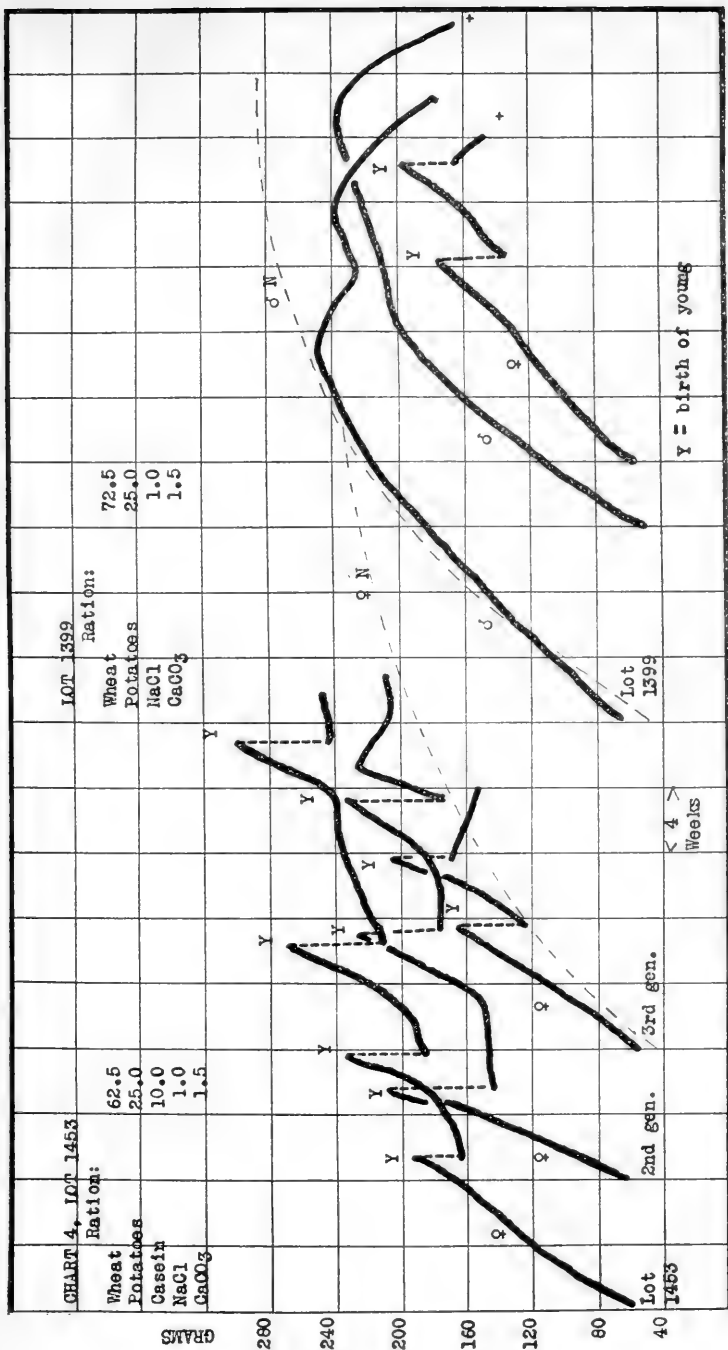
Lot 1407 contained three females. They had altogether thirty-four young (five litters) and weaned fourteen of them. Two females of the second generation were confined to this diet. One had a litter of six but they were very puny. Some of these were destroyed when very young. A litter which was nursed 11 days was then only about 60 per cent as heavy as it should have been if the mother's diet had been satisfactory.

Lot 1461 was fed millet seed, potatoes, butter fat, and casein in Period 1. Little growth was possible on this food. In Period 2 sodium chloride and calcium carbonate were added. Growth took place at a rapid rate after this addition was made. There were three females in this group. They had forty-six young (seven litters) of which thirty-one were weaned. One female of the second generation was restricted to the diet and had a litter of eleven young but killed them all, and died herself soon afterward. Other young from the original group on this diet were very inferior. Where these young were not destroyed when very small they remained stunted while nursing. This we interpret as being due to faulty composition of the milk secreted by the mother. It is probable this was the result of some toxic substance passing into the milk from the millet seed.

These records all show that millet seed when fed in liberal amounts causes injury. This we provisionally interpret as due to the presence of some toxic substance in millet seed. That a shortage of fat-soluble A was not responsible for the results observed is shown in Chart 2. Among the farmers of the middle west there is a common belief that liberal feeding of millet causes injury to farm animals. The toxicity of millet seed is still under investigation.

*Chart 4.*—The records in this chart are those of rats which were fed diets in which wheat and potatoes furnished the sole source of fat-soluble A. The protein content in the diet of Lot 1399 was approximately 9 per cent. That this amount of protein from these





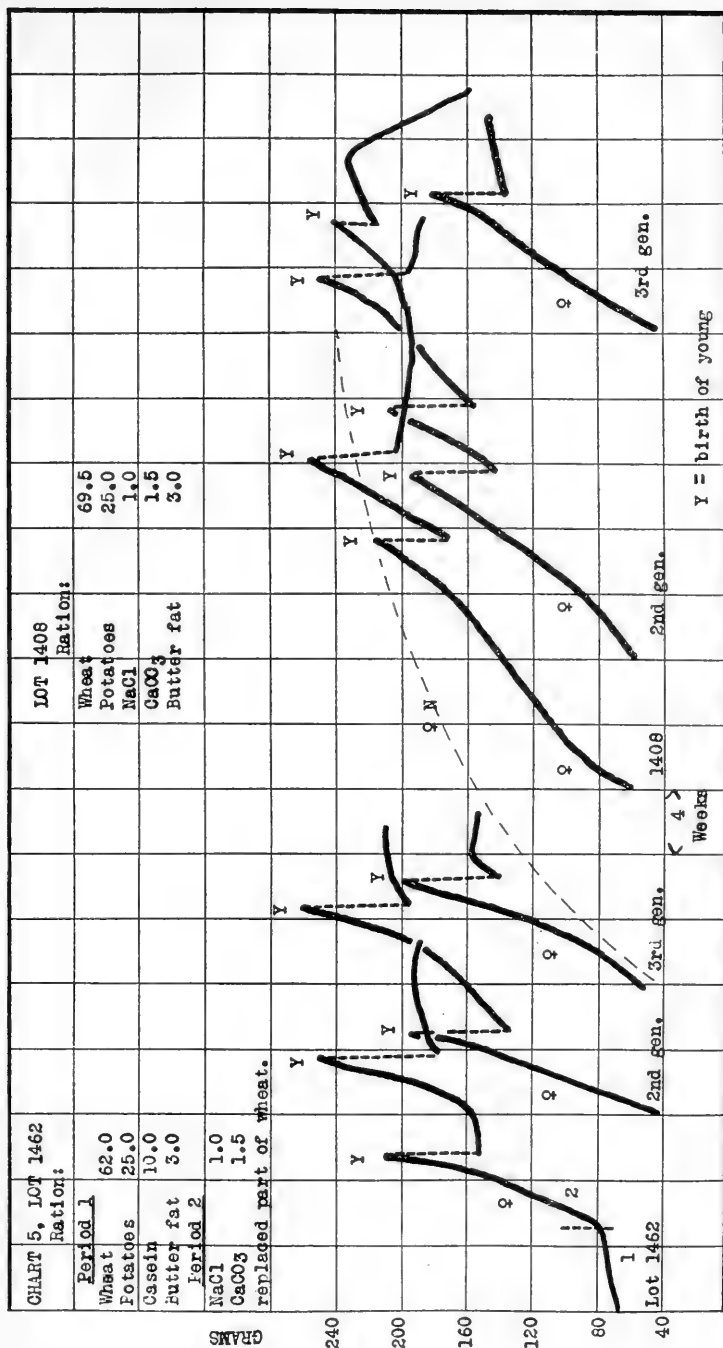
sources is capable of inducing good growth and moderately high fertility is shown by a comparison with Lot 1408, Chart 5. The limiting factor in Lot 1399 is fat-soluble A. There were two females in this group. One had two litters (eleven young) but destroyed them soon after birth. The other remained sterile.

Lot 1453 had a diet similar to that of Lot 1399 except that it contained 10 per cent of casein to supplement its protein content. This exerted marked improvement in their growth, fertility, and longevity. It is of special importance to compare these curves with those of Lots 1399 and 1408. The diet of Lot 1399 is very much improved either by the addition of protein or fat-soluble A. This illustrates a principle which many investigators have failed to grasp; *viz.*, that all the other factors in an experimental diet must accurately be evaluated before judgment can safely be formed concerning the quality of a food or preparation in respect to a factor under investigation. For example, the diet of Lot 1453 appears to contain sufficient fat-soluble A. Actually it is distinctly below the optimum in this substance. It contains the same amount of fat-soluble A as does the diet of Lot 1399, but this deficiency is not apparent in Lot 1453 for some months because of the excellent quality in all other factors. Deficiency in fat-soluble A, however, became manifest in the lowered nutritive value of the milk and caused injury to the young, especially in the third generation.

Two females in this group had collectively fifty-two young (seven litters) and weaned forty-two of them. One second generation female had twenty young (three litters) and weaned eleven. One third generation female had eleven young (two litters) and weaned nine of them. The young were strong and vigorous in most cases. A few of the second litter obtained from a third generation female were, however, quite undersized and puny.

*Chart 5.*—The curve of Lot 1462, Period 1, illustrates the behavior of young rats fed a diet in which all the inorganic salts were furnished by a mixture of wheat, casein, and potatoes. No growth could take place on this diet even though all factors other than the inorganic elements were properly constituted. In Period 2 when calcium carbonate and sodium chloride were added growth took place at once and at the maximum rate possible.

There were three females in this group. They had together forty-five young (six litters) and successfully weaned them all.



One second generation female was restricted to the family diet. She had two litters (sixteen young) and weaned them all. One third generation female had a litter of nine and reared them all. The young were always in excellent condition. This is an example of the success which we regularly see in successful growth and rearing of young when the mother's diet is satisfactory.

Lot 1408 derived all its protein from wheat and potato. The protein content of the diet was about 9 per cent, two-thirds of which was derived from wheat and one-third from potato. This amount of protein from these sources did not support growth at the maximum rate possible but the results indicate that there is some improvement in quality by combining proteins from these sources.

Two females in this group had twenty-one young collectively (four litters) and weaned eleven. One female in the second generation had eighteen young (three litters) and weaned eight of them. Two third generation females had fifteen young (two litters) of which twelve were weaned. The young in all these litters were small for their ages, and the nursing period was considerably prolonged in all cases in order to bring the young to a state of independence. Since these litters were not large in the first or second generation, it would seem probable that the failure of the young to thrive in a normal manner was due to faults in the quality rather than to lack of sufficient milk.

A comparison of the reproduction records of these groups (Lots 1462 and 1408) shows clearly that the infant mortality in the latter was the result of an inadequacy of the protein of the diet. On low protein diets, or diets containing protein of poor quality, we have regularly observed the necessity of a prolonged nursing period in order to fit the young to subsist on rations suitable for the adult.

*Chart 6.*—Lot 2150 illustrates a typical record of a group of rats fed from weaning time on a diet in which the protein was derived from peas and potatoes. Two-thirds of the protein was supplied by peas and the remainder by potato. The diet contained 9 per cent of protein ( $N \times 6.25$ ). Growth on this diet was approximately normal, showing that the mixed proteins of peas and potato at this plane of intake are far superior to pea proteins alone fed at this level (compare Lot 2376, Chart 1). Since dried potato is so





low in protein it is not possible to make a direct comparison of these mixed proteins with potato protein alone. That this content of protein from these sources is below the optimum is shown by a comparison of Lots 2150 and 2172.

There were three females in this group. They had collectively six litters (eighteen young) but none was weaned. Two other litters were born but were destroyed by the mother before the number was ascertained. The rats in this group looked very old at about the age of 1 year.

Lot 2172 had a diet similar to that of Lot 2150 except that it contained casein but no butter fat. The growth records were remarkably good, notwithstanding the fact that all the fat-soluble A was derived from peas (31.8 per cent) and potato (25 per cent). One male reached a weight of 360 gm., and a female, a non-pregnant weight of 245 gm.

There were two females in the group. They had together twenty-three young (four litters) and weaned thirteen of them. The young were inferior in appearance, but did not develop xerophthalmia. One mother, however, developed inflamed eyes. Four second generation females were restricted to the food mixture but all died after being subjected to this diet for 4 to 5 months without having any young.

Lot 1459 was fed a diet similar to that of Lot 2172, except that it contained about double the amount of peas. The first generation fed this diet grew well, but the fertility was low and they aged very early. They were ready to die at about a year.

There were two females in this group. They had collectively nineteen young (three litters) and weaned nine of them. Two females in the second generation were restricted to the diet. One became pregnant, but died while giving birth to young. The other died at the age of 6 months. This ration contained a high protein content (about 27.5 per cent), more than half of which was from peas. Pea protein when fed at high levels apparently causes damage to animals.<sup>3</sup> This diet (Lot 1459) contained considerably more fat-soluble A and protein in its content of peas than did that of Lot 2172. All other factors were equally satis-

<sup>3</sup> McCollum, E. V., Simmonds, N., and Parsons, H. T., *J. Biol. Chem.*, 1919, xxxvii, 287.

factory or to the advantage (water-soluble B) in the case of Lot 1459, yet the nutrition of the latter group was distinctly inferior to Lot 2172. These results harmonize with the observation of Osborne and Mendel<sup>4</sup> that young rats fed purified pea protein as the sole source of this factor steadily declined.

The records of Lot 1459 should be compared with those of Lot 1468 (Chart 7). The diets of these groups were similar but the latter contained more fat-soluble A, and this resulted in marked improvement in their condition.

*Chart 7.*—The rats of Lots 1414 and 1405 derived their protein entirely from peas and potatoes. The pea protein constituted about 88 per cent of the total. The protein content of these food mixtures was 17 to 18 per cent. Even at this high level of intake these proteins are inferior to a mixture derived from wheat and potato, as is shown by a comparison of these groups with Lot 1408 (Chart 5).

Lot 1414 contained two females. They had collectively three litters (nineteen young) and weaned all of them. One female of the second generation had one litter of three and weaned them. Some of the original group were kept on the diet more than 10 months but by 8 months they appeared old looking.

Lot 1405 was fed a diet closely similar to that of Lot 1414 except that it contained no butter fat. The inferiority of these animals as compared with Lot 1414 was due entirely to the lower intake of fat-soluble A.

A striking illustration of the danger of misinterpreting data obtained in nutrition experiments of this character is afforded by a comparison of four sets of records in these charts. Lot 2172 (Chart 6) derived all its fat soluble-A from 31.8 per cent of peas and 25 per cent of potato. The protein was enhanced by the addition of casein and the necessary inorganic salts were added to complete the mineral content. During the first year of life these animals appeared normal except for the high infant mortality. Lot 1459 (Chart 6) had more peas, the same amount of potatoes and casein, and the same salts. It, therefore, secured more protein and more fat-soluble A than Lot 2172, yet the development of Lot 2172 was much more satisfactory. If we did not have the records of Lot 2172 we should have made the statement from the results of Lot 1405 that peas are very low in fat-soluble A. How-

<sup>4</sup> Osborne, T. B., and Mendel, L. B., *Z. physiol. Chem.*, 1912, lxxx, 307.



ever, when the protein content was improved with casein, as in Lot 2172, the fat-soluble A derived from 31.8 per cent of peas and 25 per cent of potato had more beneficial effect than more than twice this amount taken with the same food mixture containing protein of lower biological value. The addition of butter fat (fat-soluble A) to Lot 1405, however, also made them develop distinctly better, as is shown in Lot 1414.

Lot 1405 (Chart 7) was fed 72.5 per cent of peas and therefore more fat-soluble A than either Lot 2172 or Lot 1459 (Chart 6), yet because the casein was omitted in Lot 1405 the animals were very inferior. The protein and phosphorus content of Lot 2172 is lower than that of Lot 1405, yet the growth was much better in the former than in the latter. This was due to superiority in the quality of the protein in the diet of Lot 2172, and this factor was of sufficient biological significance to make a decided difference in the well being of the two groups. These illustrate border-line cases of malnutrition. They represent a type of diet in which a small variation of quality upward in one or another factor, as protein or salts, may so modify the early history of the experimental animals as to make a particular factor, *e.g.* fat-soluble A content, appear in one set of results to be adequate for the physiological needs of the animals, while a variation in the quality downward either in protein or salts, such variation remaining at the same time well within physiological limits, may entirely change the deductions with respect to the value of the constant factor fat-soluble A.

This same idea is also brought out in Chart 8, Lot 1454, and also in Chart 11, Lot 1451 (see discussion under these charts).

Lot 1468 in Period 1 was fed a diet of peas, potatoes, casein, and butter fat. The growth of the animals on this food was in no case more than half normal (Period 1). In Period 2 sodium chloride and calcium carbonate were added. This led to a prompt response with growth and reproduction. It appears probable from these results that there is less injurious effect of high pea protein ingestion when its quality is enhanced by a casein addition than when the same pea protein content is taken without such improvement.

This group contained two females. They had collectively fourteen young (two litters) and weaned them all. Two second generation females had collectively twelve young (a litter each) and weaned eleven. These mothers were discarded after weaning

their young. These animals presented a dirty appearance and were badly stained with their own urine.

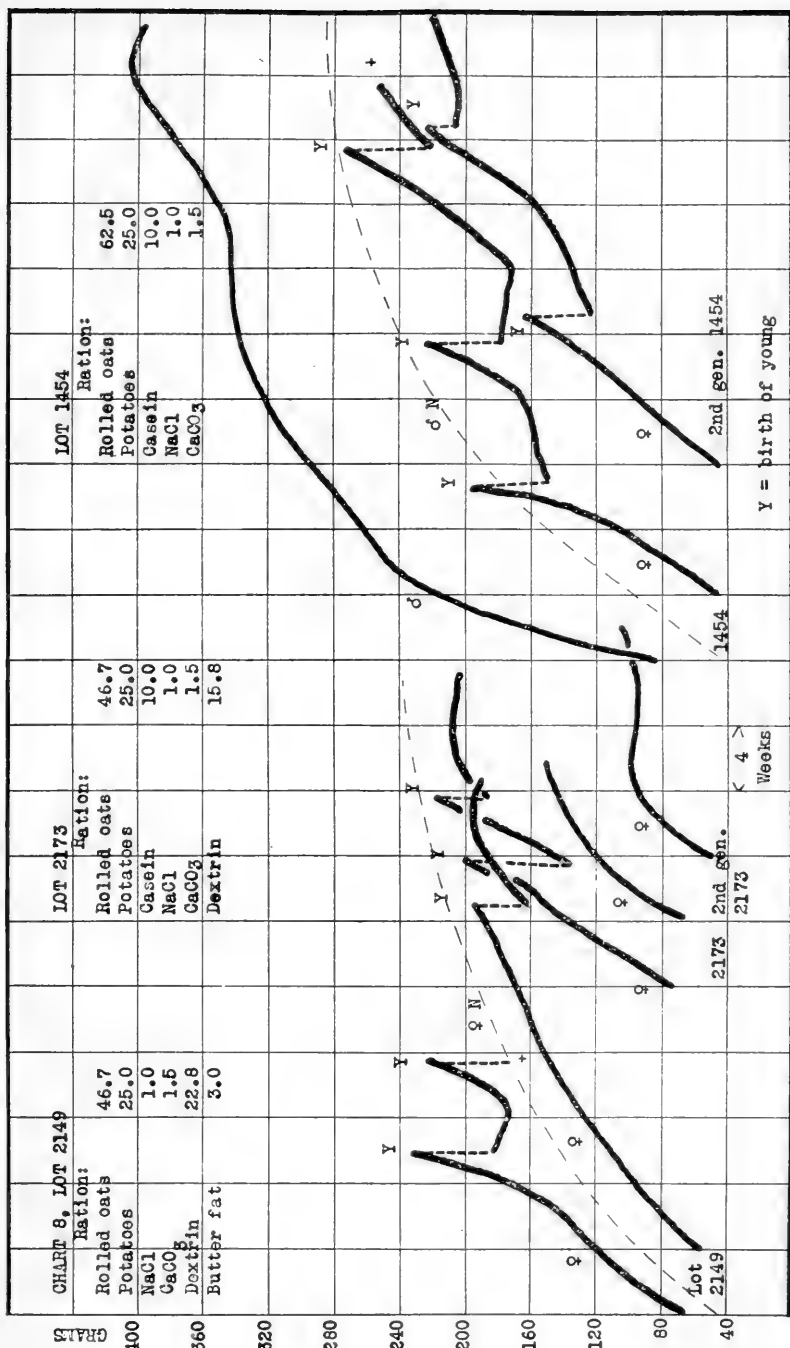
*Chart 8.*—Lot 2149 was fed a diet, the protein of which was derived entirely from rolled oats and potato. It contained 9 per cent of total protein ( $N \times 6.25$ ), two-thirds of which was furnished by oats and one-third by potato. Other factors than protein were satisfactorily supplemented. Growth on this diet was somewhat below the optimum, but the protein derived from this combination is of better quality than a similar amount of oat protein alone. This combination does not possess so high a biological value as does a protein mixture from wheat and potato in the same proportions. This is illustrated by Lot 1408 (Chart 5).

Three females grew up on this diet. They had collectively thirty-one young (four litters) but none was weaned. The males were apparently in good condition at 7 months of age, but the females presented a poorer appearance. Those young which were not killed by the mother grew so little while nursing that it is evident that the nutritive value of the milk was low.

Lot 2173 derived all its fat-soluble A from rolled oats and potato. In other respects this diet was comparable with Lot 2149. The protein in the diet of Lot 2173 was enhanced by the addition of casein.

Three females grew up on this diet. One female had two litters, another, one litter, collectively eighteen young, and weaned but four of them. A third female became pregnant but died while giving birth to her young. The young were in a very poor condition. Two daughters of these mothers were restricted to the diet but never had any young. All these animals aged early. Their span of life was only 5 to 7 months.

Lot 1454 presents remarkable growth curves on a diet which all previous experience indicated to be very poor in fat-soluble A. All of this factor was derived from 62.5 per cent of rolled oats and 25 per cent of cooked dried potato. One male reached a weight of over 400 gm. Two females each had three litters, collectively forty-seven young, and weaned twenty-six. One daughter grew up on the diet and had two litters but lost them all during the first few days. These young were in poor condition and developed xerophthalmia in some cases. At the age of 11 months the animals appeared very old, but autopsy revealed nothing pathological.



Our experience in studying the dietary properties of the oat kernel has demonstrated it to be the poorest of the cereal grains in fat-soluble A. Steenbock and Gross report the white potato to be an inadequate source of this factor.<sup>5</sup> In interpreting the value of this diet with respect to fat-soluble A, Lot 1454 (Chart 8) and Lots 1409 and 1400 (Chart 9) should be compared. On the diet of Lot 1400 but little growth could take place, and xerophthalmia developed. Lot 1409, whose diet differed from that of Lot 1400 only in containing butter fat, was able to grow and reproduce. This was due specifically to the increased content of fat-soluble A. Lot 1454, whose diet differed from that of Lot 1400 only in that its protein content was improved by the addition of casein was also able to grow normally and reproduce and rear some young. The infant mortality was higher than that of Lot 1409. The content of fat-soluble A was the same in the diets of Lots 1400 and 1454. Such experimental results as these show the fallacy of deductions such as have been made by Hess and Unger<sup>6</sup> who fed infants during several months on a diet somewhat low in fat-soluble A but of excellent quality in other respects, and concluded that the factor fat-soluble A is not of great importance in practical human nutrition. Judgment on this matter must be based on a full appreciation of the difference between border-line malnutrition with its attendant grave dangers from infections or unfavorable reaction to any chance modification of the diet so as to reduce its quality in any way.

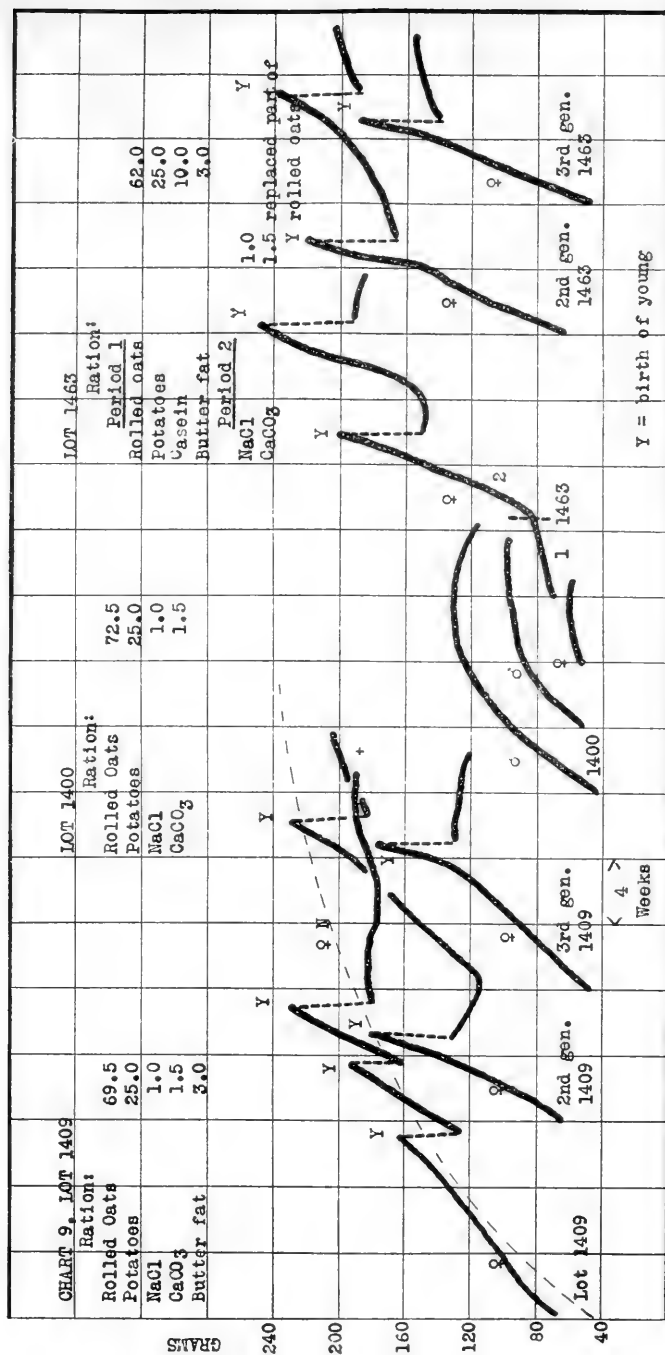
*Chart 9.*—Lot 1409 was fed a diet in which the protein was all derived from rolled oats and potato. The content of protein in the food mixture was about 12.5 per cent, about 16 per cent of this being derived from potato, and 84 per cent from oats. All other factors were adequately supplemented.

The rats which were confined to this diet never grew to the normal adult size. There were two females in this group. They had collectively eighteen young (four litters) but only two were weaned. The mothers destroyed the young. One of these rats died during the birth of her third litter. One second generation female grew up on the family diet and had fifteen young (two litters) and

<sup>5</sup> Steenbock, H., and Gross, E. G., *J. Biol. Chem.*, 1919, xl, 501.

<sup>6</sup> Hess, A. F., and Unger, L. J., *J. Am. Med. Assn.*, 1920, lxxiv, 217.





weaned thirteen of them. One third generation female had one litter of six and successfully weaned them.

Lot 1400 was fed a diet like that of Lot 1409 except that it did not have a supplemental addition of fat-soluble A. Growth on this diet was slow and incomplete. The rats all died in 4 to 5 months after being placed upon the ration. Xerophthalmia developed in three of the four rats confined to this food mixture.

This diet with added fat-soluble A is capable of inducing nearly normal growth and supports reproduction and rearing of some young (Lot 1409). This ration with added protein has induced the maximum amount of growth in rats and supported reproduction and rearing of some young, although the infant mortality was high (Lot 1454, Chart 8). This illustrates the fact that one is not justified in drawing conclusions concerning the content of fat-soluble A in a foodstuff without furnishing evidence that all factors in the diet are accurately evaluated.

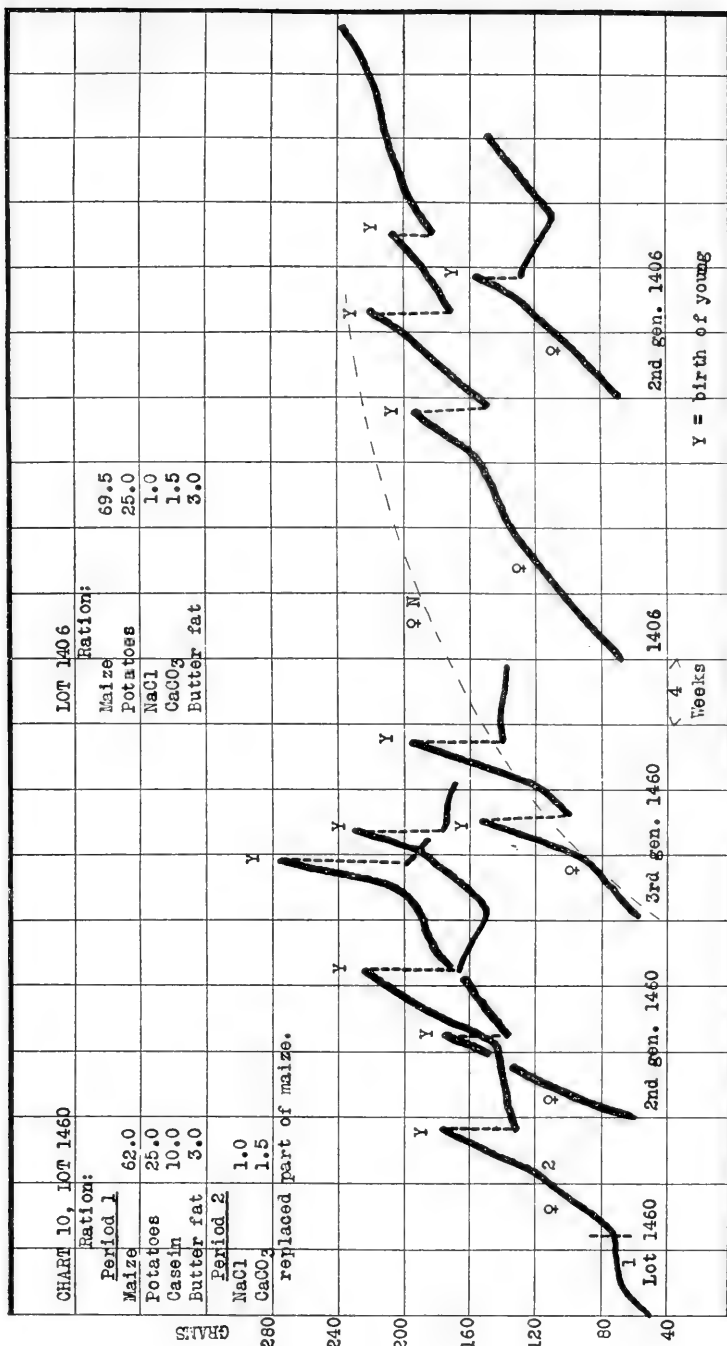
Lot 1463 was fed in Period 1 a diet consisting of rolled oats, potatoes, casein, and butter fat. The inorganic content of the diet was entirely derived from these sources. Practically no growth could take place on this food. In Period 2 sodium chloride and calcium carbonate were added. This led to marked response with growth to the full adult size.

Three females in this group had collectively thirty-eight young (six litters) and weaned thirty-six. One second generation female was restricted to the diet. She had fifteen young (two litters) of which fourteen were weaned. One third generation female had a litter of nine and weaned them all. These young were in all cases in excellent nutritive condition.

A comparison of the reproduction records and infant mortality of Lot 1463 with those of Lot 1454 (Chart 8) shows clearly the beneficial effects of a liberal supply of fat-soluble A. This is shown by a mortality among the young of 45 per cent in Lot 1454 in the first generation and of 100 per cent in the second generation.

In Lot 1463, 95 per cent of the young were weaned. The appearance, size, and condition of the young of these two groups presented as marked a contrast as did the infant mortality.

*Chart 10.*—Lot 1406 derived all its protein from 69.5 per cent of maize and 25.0 per cent of potatoes. All other factors were



satisfactorily adjusted by suitable additions. Growth was somewhat retarded because of the quality in the protein factor.

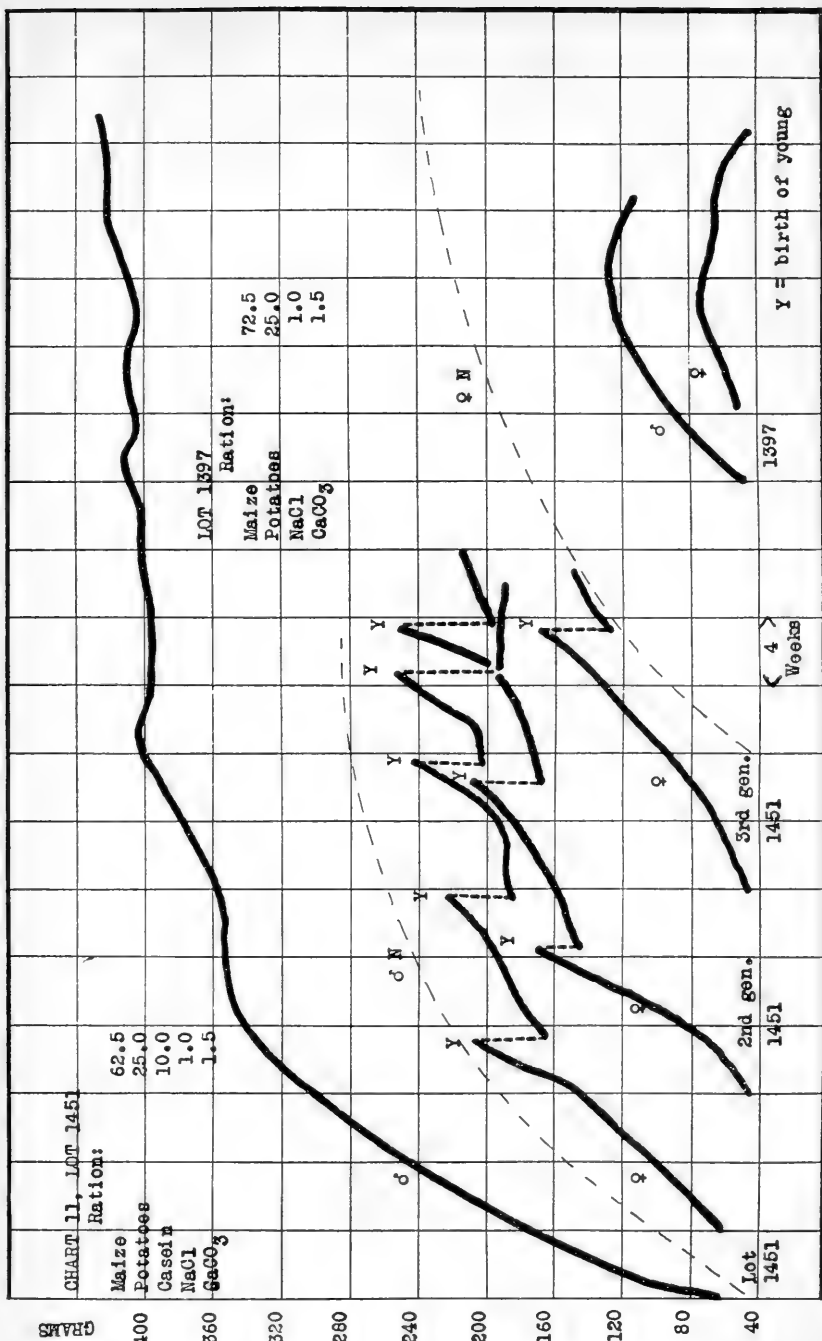
Three females grew on this diet. They had collectively thirty-five young (six litters) and weaned eleven of them. Two second generation females were restricted to this diet. One had a litter of two young and after a long nursing period weaned them in poor condition. The other remained sterile. The animals in this group were always somewhat undersized and of poor appearance.

Lot 1460 in Period 2 had a diet like that of Lot 1406 except that it contained 10 per cent of casein. In Period 1 no inorganic salts were added. Without these growth was suspended, but with them the animals grew rapidly. This shows that in Lot 1406 the quality and amount of protein was the limiting factor.

Three females grew up on this diet. They had collectively fifty young (seven litters) and weaned forty-eight of them in good condition. One second generation female had seventeen young (two litters) and weaned fifteen of them. One third generation female had fifteen young (two litters) and weaned seven. The young were vigorous and active but their coats were not as glossy and well kept as those of other rats which we have observed. The contrast between these young and those of Lot 1406, on a lower protein plane, was very marked.

*Chart 11.*—Lot 1397 derived both protein and fat-soluble A entirely from maize and potato. The maize used in these experiments was of the yellow variety. A comparison of these growth curves with those of Lot 1451 (Chart 11) shows that notwithstanding the high content of yellow maize in this food mixture, protein was the first limiting factor in preventing normal development. Lack of sufficient fat-soluble A was, however, a very important deficiency in this diet, as shown by comparing the curves of Lot 1397 with those of Lot 1406 (Chart 10).

There was more fat-soluble A in the diet of Lot 1397 than in that of Lot 1451, yet the satisfactory content of protein in the latter diet made it appear, in the absence of a complete series of biological tests, to contain a satisfactory amount of this factor. Although certain yellow maizes doubtless contain more fat-soluble A than certain white varieties it seems to us that there has been some exaggeration of the value of yellow maize as a source of this



factor.<sup>7</sup> If we were limited to experiments with diets of the type fed Lots 1451 (Chart 11) and 1454 (Chart 8) we could easily have fallen into the error of interpreting the results as indicating that diets containing 60 to 70 per cent of yellow maize or even rolled oats supplemented with potato to the extent of 25 per cent of the food mixture would prove satisfactory in their content of fat-soluble A. More carefully planned experiments eliminate the danger of error in studies of this nature.

Lot 1451 contained two females. They had collectively sixty-four young (eight litters) and weaned fifty-nine. Two second generation females had five litters (twenty-seven young) and weaned twenty-two. Two third generation females had collectively eleven young (two litters) and weaned ten. All the young were in good condition, but in a few cases the hair was short and silky in appearance.

<sup>7</sup> Steenbock, H., and Boutwell, P. W., *J. Biol. Chem.*, 1920, xli, 81.

## SUPPLEMENTARY PROTEIN VALUES IN FOODS.

### IV. THE SUPPLEMENTARY RELATIONS OF CEREAL GRAIN WITH CEREAL GRAIN; LEGUME SEED WITH LEGUME SEED; AND CEREAL GRAIN WITH LEGUME SEED; WITH RESPECT TO IMPROVEMENT IN THE QUALITY OF THEIR PROTEINS.

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(Received for publication, March 21, 1921.)

Those who have discussed the world's food supply during the past few years have talked in great measure about the cereal grains as the most important and economical source of human food. Indeed, for many years the cereals and legume seeds have been increasingly cultivated both in Europe and America to meet the needs of their rapidly increasing populations. Bread grains have actually become the staff of life of a large part of the human family. It is now recognized by students of nutrition that the cereal grains individually and collectively are incomplete foods.<sup>1</sup> The war has taught through tragic experience that children cannot be kept alive for a very long period on such a food supply, although the requisite calories and energy may be supplied. The history of scurvy furnishes numerous examples of the dangers attending the restriction of the diet of adults to cereal products, legume seeds, and meats. Nevertheless, statisticians and persons in charge of feeding large groups of people in armies, prisons, asylums, hospitals, and labor camps, still in many instances show lack of appreciation of the dangers of limiting people to certain types of restricted diets.

It was natural that in the early efforts to discover the fundamental truths regarding the nutritive requirements of mammals, inves-

<sup>1</sup> McCollum, E. V., *The newer knowledge of nutrition*, New York, 1918. Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1920, xli, 275.

tigators should have attached much importance to the rate of growth during limited periods by young animals fed diets of different types. It was spectacular to observe complete failure in one case on a diet with which a chemist could find nothing amiss, and successful growth for weeks or months on another diet which according to older views should have been no more satisfactory than the first. With advancing knowledge, however, it became possible to interpret the cause of failure or success with any given diet, and new goals were set up by those who were able to make progress in nutrition studies. In our laboratory we have for several years sought to approach the solution of the problem of what constitutes the optimum diet for the purpose of promoting growth, of supporting highest fertility, greatest success in rearing young, and of preserving for as long a period as possible the characteristics of youth, and of extending to the extreme limit the span of life. With this objective we have come, as the result of much experience, to question whether the extension of the use of cereal grains in the diet of man has not already passed the limit of safety. At least it is more necessary now than formerly that the remaining components of the diet should be chosen with knowledge and care in order to correct the deficiencies of the cereal, tuber, and muscle meat mixture, or the bread, potato, and meat type of diet which has become so prominent a feature of the nutrition of many American and European families at the present time. It has been already pointed out that the cereal grains, legume seeds, and muscle tissue meats are too poor in calcium, sodium, chlorine, and fat-soluble A to meet the physiological requirements of a mammal. Mixtures of these in any variety are little, if any, better sources of the essential mineral elements or of fat-soluble A than are the individual foods themselves.<sup>2</sup>

Primitive man ate everything he could secure which was edible. His animal food included the flesh of such game as he could catch, and also fish, eggs, birds, shell-fish, insects, etc. Among the vegetable products which he doubtless ate were fruits, berries, fleshy roots, nuts, and a few other seeds of plants, among which were the seeds of those grasses which have since been developed into our

<sup>2</sup> McCollum, E. V., *Proceedings of the Institute of Medicine of Chicago*, 1920, iii, 13.



cereal crops. There are relatively few regions where nuts are sufficiently abundant to furnish a regular article of diet for a sparse population during even a few months in the year, and the supply of cereal grains was even more inadequate. The cereal grains are the seeds of several grasses. In a country where no agriculture was practised, grasses would be cropped by grazing animals and the development of seed greatly interfered with. As Huntington points out, extensive agriculture was impossible until after animals were domesticated.<sup>3</sup> Such seed as was produced was born on isolated and scattered stems, and would be difficult to harvest in appreciable quantities. One exception was the wild rice plant which grew in the water and was therefore protected to some extent from grazing animals. It was more abundant in certain places than the seeds of any land grasses were likely to be in the unmolested fruiting condition. Rice was therefore harvested from very early times in Asia and in some of the northern states of America. Even in these favored regions of shallow lakes and rivers, however, rice never formed a principal constituent of the diet of the Indians, but only an adjuvant in the fall and early winter. Grass seeds of the type of the cereal grains are always eagerly sought for at ripening by birds, and the harvest time would naturally be short. Maize was never a prominent article of diet among the Indians, but only served to vary their otherwise carnivorous food supply. All the higher apes eat more or less of tender leaves which have mild flavors. After man reached a stage of development where food was regularly cooked he was able to eat coarse vegetables of the leafy type in greater variety and in larger amounts than when he had to eat them raw, because of the difficulty of digesting some of them. Pot-herbs early became a regular part of the diet of man as he advanced toward civilization. They are today the outstanding feature of the diet of the Chinese and Japanese.

The great increase in the consumption of cereal grains in various forms as flours, corn-meal, corn grits, rolled oats, and in the form of the many breakfast foods found on the market as human food is an incident in connection with the development of modern industry, and the change from a rural to an urban life. This has forced a great part of the population to depend upon the

<sup>3</sup> Huntington, E., *Civilization and climate*, New Haven, 1916.

remainder for food while they operate the machinery of industry. The land yields calories and protein in greatest abundance when farmed to cereals and leguminous plants, rather than when used for the production of milk or meat. This type of farming has therefore been encouraged, and wheat and maize production has been stimulated year by year to higher and higher levels until the world's capacity has nearly been reached in this respect. This great consumption of grains has changed the character of the diet profoundly from what it has ever been before in human history, and in a manner which tends to undermine the vitality. The national dietetic sin of America and many parts of Europe has grown to be close adherence to a meat, bread, and potato diet, or other foods which have similar dietary properties. When it is remembered that the cereal grains are now all but universally decorticated and degerminated for the purpose of producing products which can be kept without commercial hazard, and that these are decidedly poorer in their dietary properties than are the seeds from which they were milled, the situation can easily be appreciated. The diet of cereals, muscle tissue meats, and tubers (the meat, bread, and potato type) is not satisfactory for the nutrition of man or animals, and is of distinctly poorer quality when highly milled products are used in abundance.<sup>4</sup>

We wish to emphasize in connection with this study of the manner in which the cereal grains supplement, or rather fail to supplement, the proteins of other cereal grains and legume seeds, the *fact that the widespread use of this class of foods represents an innovation in man's diet, and one which is not for the best.* The cereals may well be used as articles of human food, but it is wiser to utilize more of the land for the extension of the dairy industry in order to increase the supply of milk and other dairy products, than to seek to extend as far as possible the production of crops which yield the greatest returns in such food units as the chemist has long recognized, but which fall short of the requirements of mammals in respects which we have but recently been able to appreciate.

When one studies the charts described in this paper it is difficult to avoid the conclusion that a diet composed too largely of cereal

<sup>4</sup> McCollum, E. V., Simmonds, N., and Parsons, H. T., *J. Biol. Chem.*, 1919, xxxviii, 113.

products, and not satisfactorily supplemented by foods which correct their deficiencies, will tend to lower the vitality and promote the early development of senile characters. We cannot agree with Daniels and Nichols that the consumption of legume seeds such as the soy bean should be increased.<sup>5</sup> It is much better to use these seeds for feeding dairy cows, for their deficiencies can be made good by the latter through the consumption of forage plants, and used as a source of milk formation. Milk forms the most satisfactory corrective food to make good the deficiencies of the cereal, muscle meat, and tuber diet now in such widespread use.

#### SUMMARY.

The charts presented in this paper bring out the following facts: Earlier observations on the inferior character of the proteins of the legume seeds have been confirmed. It is further shown that protein mixtures derived from two such seeds, the selection being made from navy or soy beans or peas, are little, if any, better than the proteins of the individual seeds, which compose the mixture when fed as the sole source of nitrogen.

Two cereal grains when combined fail to form a protein mixture which is markedly superior to the same amount of protein from a single grain for the nutrition of the rat.

In certain instances the improvement in the quality of the proteins is decidedly great when a cereal grain is supplemented with a legume seed. Conspicuous examples of such enhancement of proteins are wheat and navy beans (Chart 12), and wheat and peas (Chart 13).

Table 1 presents the records of the weights of several litters of young rats, the mothers of which were confined to diets which were essentially comparable in quality in all factors other than protein (Lots 2369, 2365, 2370, and 1236). The rate of growth of the nursing young served as an index to the extent to which the protein moiety of the food mixture met the needs of the mother for her own maintenance and for milk production.

Lot 2369 should be contrasted with Lot 2069. Each litter contained four young. Lot 2369 derived its protein (9 per cent) from maize and peas. Growth was retarded, indicating that the quality of the milk was below the optimum. Lot 2069 derived its

<sup>5</sup> Daniels, A. L., and Nichols, N. B., *J. Biol. Chem.*, 1917, xxxii, 91.

TABLE I.

This table shows typical records of nursing mothers on diets containing 9 per cent of protein from different sources, as contrasted with others on diets equally well constituted in respect to all other factors and containing higher protein contents of good quality.

Lot No.	Weight of ♀ when young were born.		Weight of ♀ 10 to 18 days later.		Weight of ♀ when through nursing.		No. of young.	Weight.						Source and content of protein.
	gm.	days	gm.	days	gm.	days		days	gm.	days	gm.	days	gm.	
2369	144	18	150	46	120	4	18	71	31	107	46	123	Maize 6 per cent protein, peas 3 per cent protein (see Chart 9).	
2069	215	10	232	22	205	4	10	100	22	197			Degerminated cereals, peas, beans, steak, and cabbage (18.2 per cent protein).	
2365	173	18	157	45	146	5	18	70	30	102	45	210	Barley 6 per cent protein, navy beans 3 per cent protein (see Chart 8).	
2370	192	12	170	37	170	5	12	74	33	160	37	175	Wheat 6 per cent protein, peas 3 per cent protein.	
2153	175	15	182	28	192	5	15	137	28	277			Degerminated cereals, peas, beans, steak (19 per cent protein).	
2370	177	20	145	44	175	7	12	81	33	167	44	250	Wheat 6 per cent protein, peas 3 per cent protein (see Chart 13).	
1236	142			40	146	7	16	133	30	286	40	544	Steak 50 per cent, salts and butter fat, 35 per cent protein.	

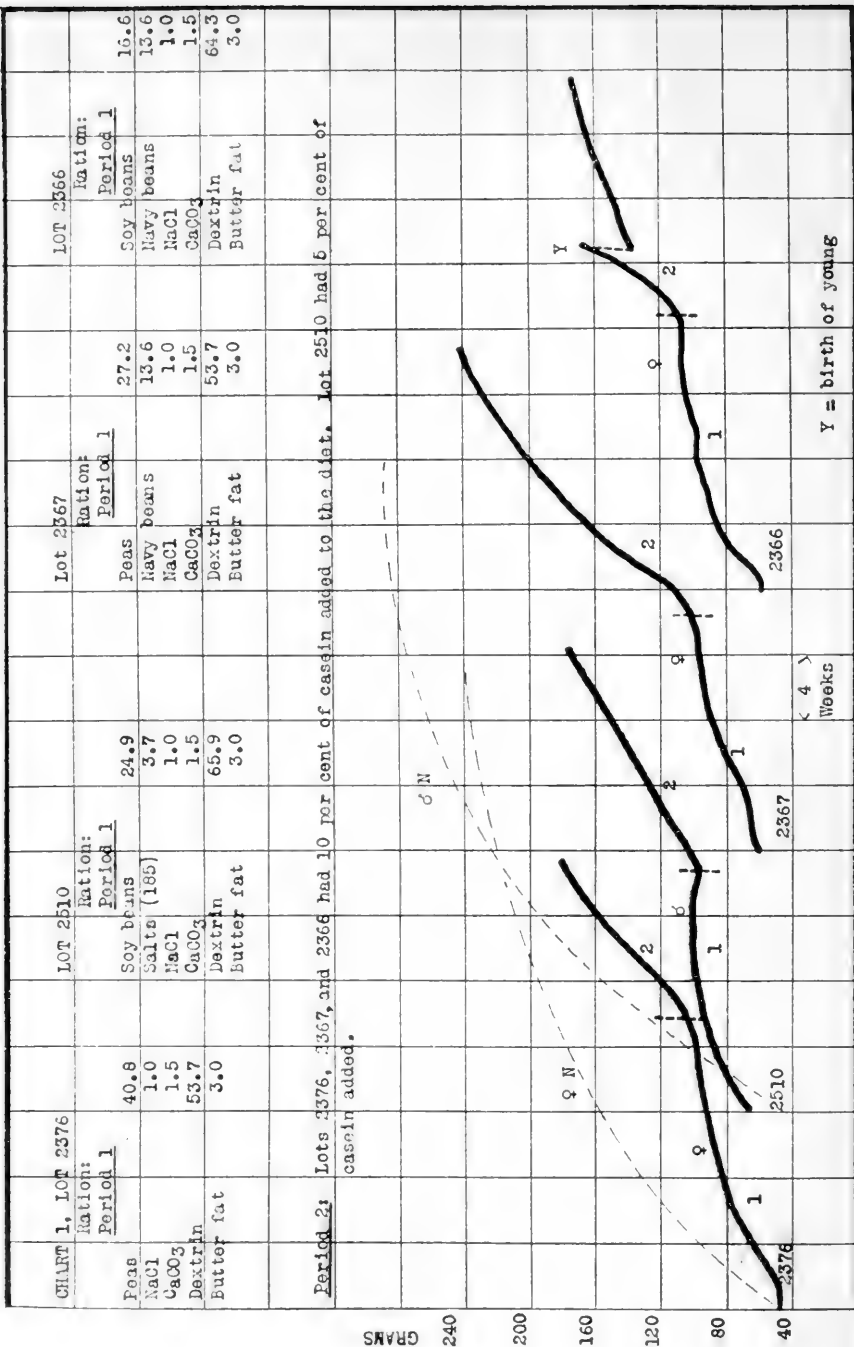
Lot 2069.	Lot 2153.	Lot 1236.
Bolted flour..... 30.0	Bolted flour..... 30.0	Round steak
Corn-meal..... 15.5	Corn-meal..... 19.5	(cooked)..... 50.0
Rice..... 8.0	Rice..... 9.5	NaCl..... 1.0
Rolled oats..... 8.0	Rolled oats..... 9.5	KCl..... 1.0
Peas..... 8.0	Peas..... 9.5	CaCO <sub>3</sub> ..... 1.5
Navy beans..... 8.0	Navy beans..... 9.5	Dextrin..... 43.5
Round steak	Round steak	Butter fat..... 3.0
(cooked)..... 10.0	(cooked)..... 10.0	
Cabbage (dry)... 10.0	NaCl..... 1.0	
NaCl..... 1.0	CaCO <sub>3</sub> ..... 1.5	
CaCO <sub>3</sub> ..... 1.5		

protein from a variety of sources, and the amount was approximately double that in the diet of Lot 2369. These young were more than 25 per cent heavier at 10 days of age than those of Lot 2369 were at 18 days.

The nursing mother in Lot 2365 derived her protein (9 per cent) from barley and navy beans. The young nursing this mother fell far behind the growth of those which nursed a mother of Lot 2370, which derived its protein (9 per cent) from wheat and peas. The former litter of five young at 18 days weighed collectively 70 gm. The latter at 12 days weighed 74 gm. This difference in the nutrition of these nursing young was solely due to the difference in quality of protein in the two food mixtures. Other equally interesting comparisons can be seen in the table.

Lots 2069 and 2153 received 18 and 19 per cent of protein, respectively, and from a variety of sources. Their diets were, however, decidedly below the optimum in their content of fat-soluble A, and for this reason would not serve to maintain the vitality of a family through successive generations. A further discussion of this type of diet and its effect on quality of milk secreted and on the span of life will be given in a later paper.

*Chart 1.*—The curves presented in this chart are typical of groups of rats which were fed from an early age on diets which derived their protein contents from: (1) peas; (2) soy beans; (3) peas and navy beans; and (4) soy beans and navy beans. In each case the content of protein in the diets was 9 per cent. Lot 2367 derived two-thirds of its protein supply from peas and the remainder from navy beans. Lot 2366 derived two-thirds of its protein from soy beans and one-third from navy beans. Deficiencies in the



mineral and fat-soluble A content of the seeds were corrected by suitable additions, and accordingly the character of the growth curves depended on the quality of the protein.

These records show that combinations of proteins of peas and navy beans or combinations of proteins of soy beans and navy beans, are not superior in their biological values to the proteins of any one of these legume seeds when fed as the sole source of protein in diets of closely comparable composition in all respects other than the source of protein.

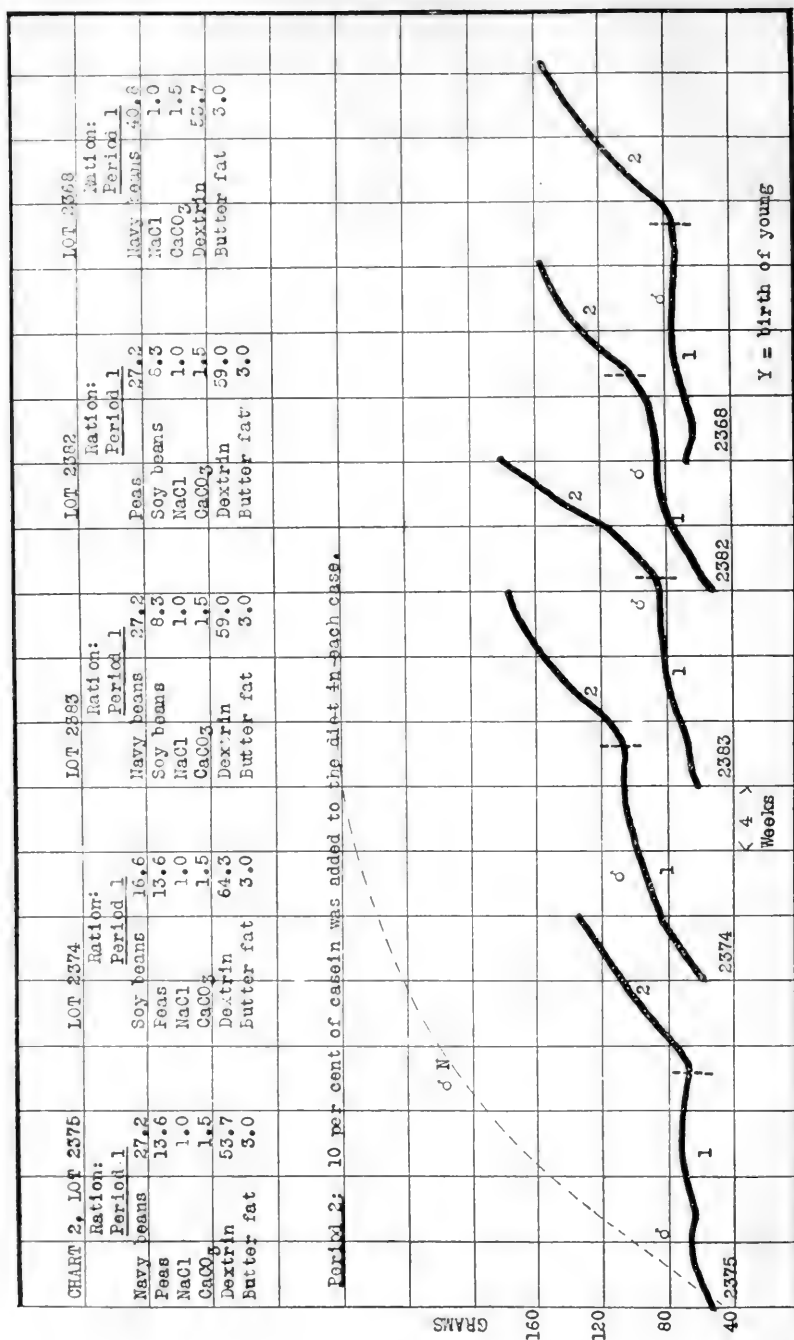
Of the females in Lot 2366 only one ever had any young. This rat had a litter of four which were destroyed soon after birth.

*Chart 2.*—The curves shown in this chart represent typical growth records of several groups of rats fed on diets deriving their proteins in each case from two legume seeds. The combinations include navy beans and peas; soy beans and peas; and navy beans and soy beans. Lot 2368 contained navy bean proteins as the sole source of nitrogen in a diet otherwise comparable in all respects to the diets of the animals whose curves are shown in the chart.

In Period 2, 10 per cent of casein was added to the diets. In every case this led to a marked response with growth, but doubtless at a slower rate than would have been the case if the animals had not suffered a long period of stunting.

It is a most remarkable fact that the legume seeds when combined with each other do not form protein mixtures which are superior to the proteins of the individual seeds themselves. This is, apparently, to be explained on the assumption that a certain amino-acid which is present in such small amount as to be the limiting factor in determining the biological value of the proteins of these seeds, is the same in each of the legume seeds used in these experiments. Otherwise, it seems that a supplementary effect should have been observed in some of these combinations. It is by no means demonstrated that all the indispensable amino-acids have been identified, but if one may judge from the recorded data relating to yields of various amino-acids it seems suggestive that the low content of cystine yielded by all legume proteins may be the explanation for their failure to enhance each others values when combined.<sup>6</sup> We shall later discuss experimental data shedding light on this point.

<sup>6</sup> Sure, B., *J. Biol. Chem.*, 1920, xliii, 443.





*Chart 3.*—The curves in this chart illustrate the growth and reproduction records of rats fed proteins derived from two cereal grains. All factors other than protein were made satisfactory by suitable additions. In all cases the protein content of the diet was 9 per cent.

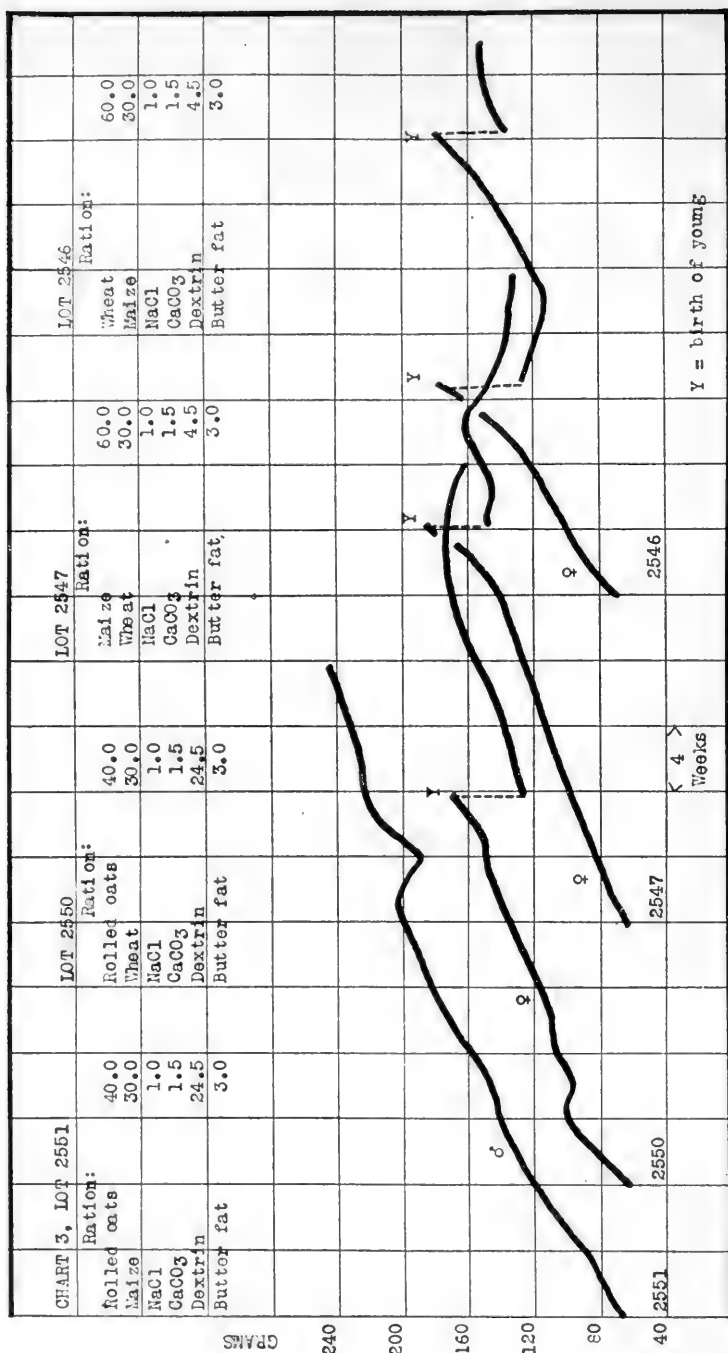
Lot 2551 derived 6 per cent of protein from rolled oats and 3 per cent from maize. Growth was only fairly satisfactory. All remained undersized. The females reached non-pregnant weights of about 150 gm. There were two females in the group but neither had any young.

Lot 2550 derived 6 per cent of protein from rolled oats and 3 per cent from wheat. These animals were undersized and not in very good condition. Three females had eleven young (two litters) but all died during the nursing period. One litter of five weighed 52 gm. at 10 days of age and was, therefore, stunted and in poor condition. At 19 days they were all dead. The stunted condition and early death of these young were probably due to the poor quality of the milk of the mother.

Lot 2547 derived 6 per cent of protein from maize and 3 per cent from wheat. These rats were undersized and their fertility was low. The growth curves of this group were not so satisfactory as we have regularly seen in animals fed a comparable diet containing 9 per cent of protein derived entirely from wheat, but were slightly better than could be secured with 9 per cent of maize protein. There were two females in this group. One remained sterile. The other had a single litter of five young, but destroyed them within a week.

Lot 2546 secured its protein entirely from wheat (6 per cent) and maize (3 per cent). While the growth curves were but slightly better in general than those of Lot 2547, the animals were in somewhat better condition.

There were two females in this group. One was sterile, while the other had but ten young (two litters) and weaned two. The nursing period was prolonged to 73 days before the young were sufficiently developed to make it possible for them to lead an independent existence on the family diet. At the age of 73 days the litter of five had been reduced to two and these weighed together but 103 gm. and were very inferior.



*Chart 4.*—Lot 2346 was fed a mixture of wheat and oat proteins in such proportions that each cereal contributed  $4\frac{1}{2}$  per cent of protein to the diet. These animals grew decidedly better than did those of Lot 2550 (Chart 3), which had the same protein intake but derived to the extent of two-thirds of the total from rolled oats. In oat and wheat mixtures the protein appears to be of better quality the higher the proportion of wheat.

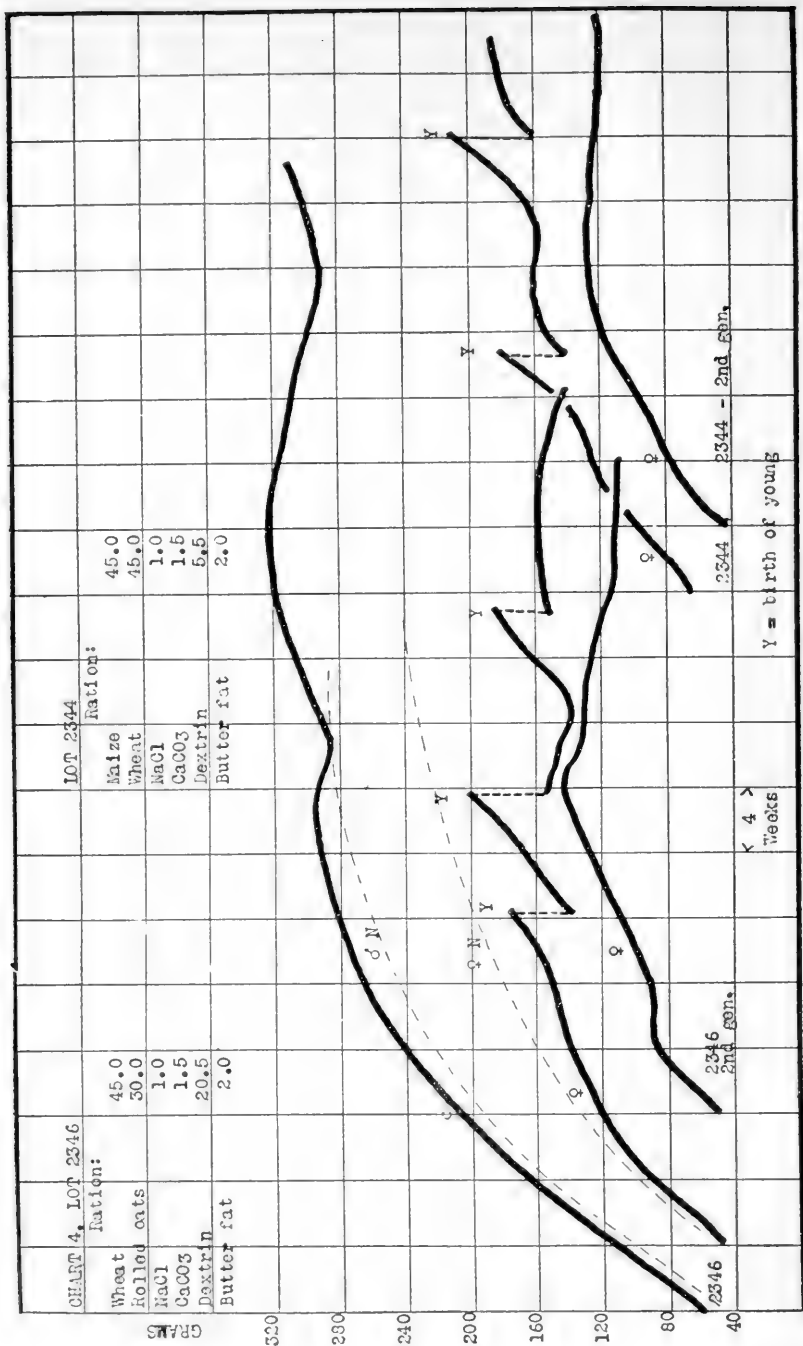
There were two females in this group. They had together thirty-three young (seven litters) and weaned nine of them. Three second generation females remained sterile on this diet. The fertility records of this group were as much superior to those of Lot 2550 as were the growth curves. The nursing period here was again long. A litter of seven young was reduced to five by 13 days. The remaining five weighed collectively at this time 48 gm. At 32 days the five weighed 101 gm. and were in rather poor condition. At 48 days the five weighed 148 gm.

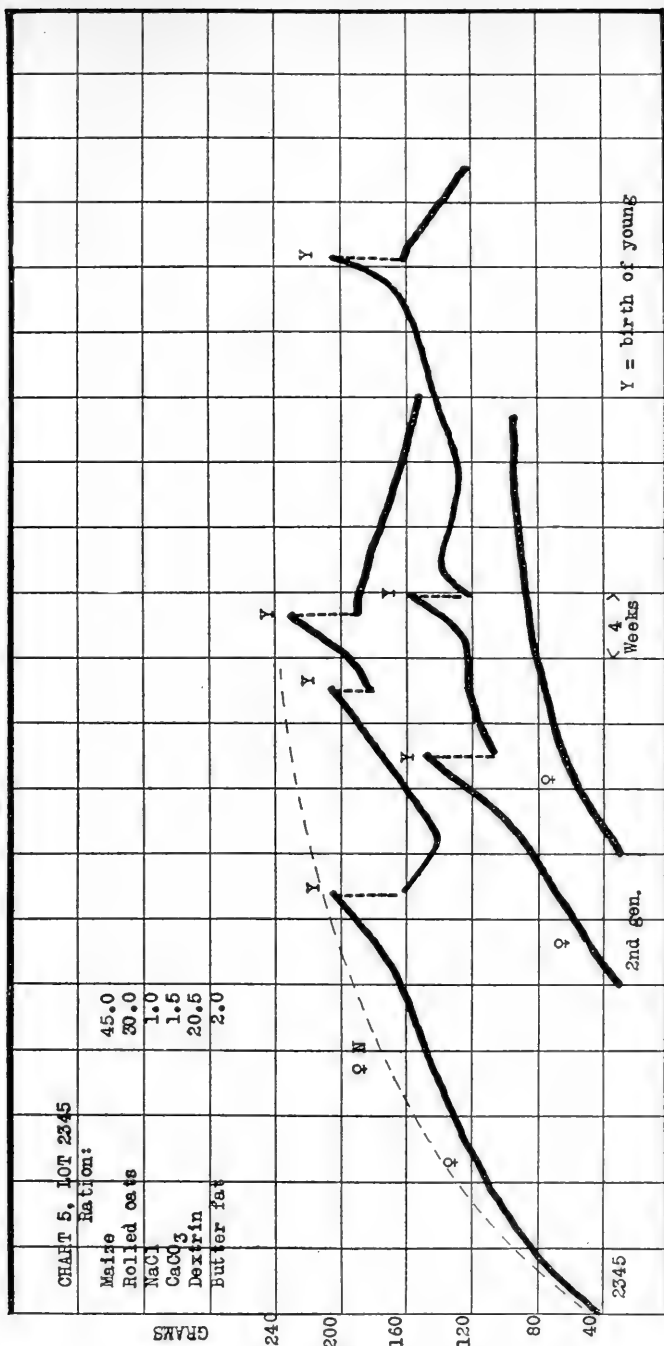
Lot 2344 was fed 9 per cent of protein derived from wheat and maize in equal proportions. The curves are better than those of Lot 2547 (Chart 3) which had 6 per cent of maize and 3 per cent of wheat proteins, respectively.

Two females had sixteen young (three litters) and weaned seven of them. These were very small for their ages. Three young at 32 days weighed 62 gm. and were poorly nourished. Two females in the second generation remained sterile.

*Chart 5.*—Lot 2345 derived its protein (9 per cent of the diet) from maize and rolled oats in equal proportions. This mixture was somewhat better than that of Lot 2551 (Chart 3) in which the oats and maize supplied 6 and 3 per cent of the protein, respectively.

Two females had thirty-five young (six litters) and weaned ten. Two other litters were born but their number were not ascertained because their mothers destroyed them as soon as they were born. One second generation female had fifteen young (three litters) and weaned three. The other remained sterile. The nursing period was very long. A litter of eight young was reduced by death to four by the 13th day. The remaining four weighed but 35 gm. At 62 days the three of these which remained weighed 92 gm. collectively. They were in an inferior condition.





Charts 1 to 5 make it clear that there is very little improvement effected in the quality of the proteins by combining two cereal grains or two legume seeds. This is so definite and striking that it forms an important contribution to the solution of the practical problems of nutrition. In Charts 6 to 13 it will be shown that in certain cases marked enhancement of the quality of the proteins is effected by making suitable combinations of a cereal grain with a legume seed.

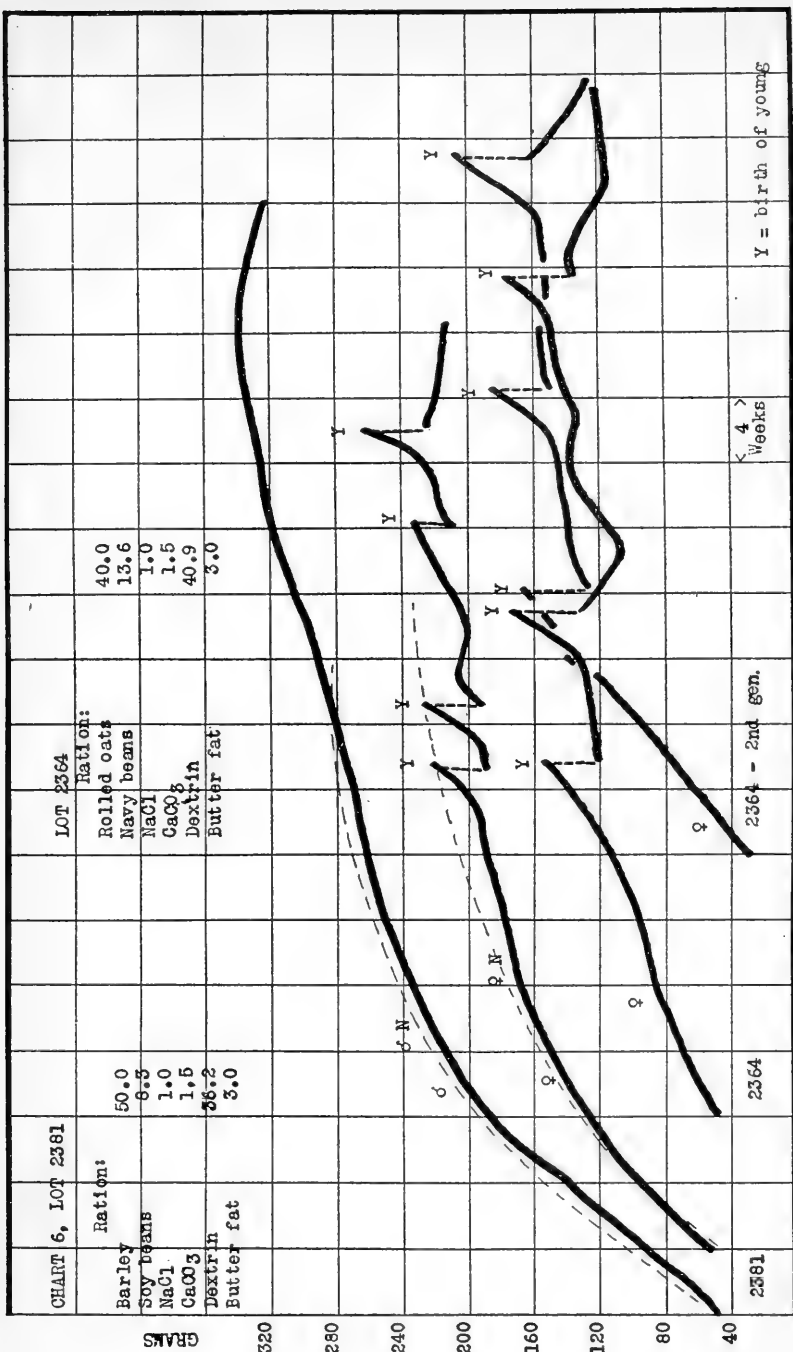
*Chart 6.*—Lot 2381 derived its proteins (9 per cent of the food mixture) from barley (6 per cent) and soy beans (3 per cent). The growth curves were normal, and were therefore decidedly superior to any which we have secured with similar diets containing 9 per cent of barley proteins. 9 per cent of soy bean proteins induce but little growth when fed as the sole source of nitrogen (Chart 1, Lot 2510). These animals were still vigorous at 12 months but looked old at 17 months of age.

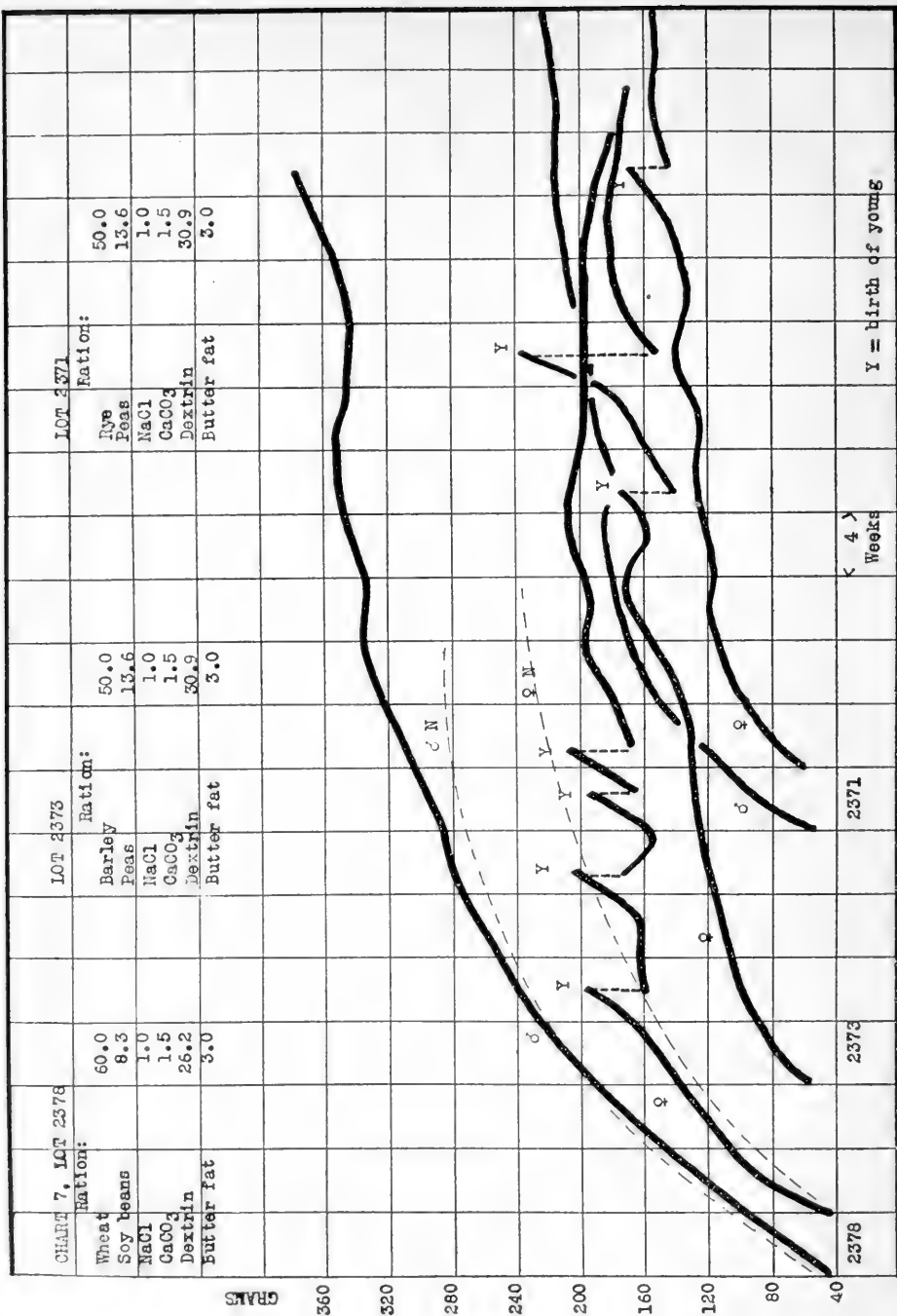
There were two females in the group. One was sterile, but the other had sixteen young (four litters), but did not wean any of them.

Lot 2364 was fed 9 per cent of protein derived from rolled oats (6 per cent) and navy beans (3 per cent). They remained undersized but their condition was better than that of rats fed a similar diet with but 9 per cent of oat protein as the sole source of nitrogen.

There were two females in the group. One died after  $4\frac{1}{2}$  months on the diet. The other had sixteen young (three litters) and weaned twelve. Six second generation females had thirty-eight young (seven litters) and weaned sixteen. Two third generation females were kept on the diet  $4\frac{1}{2}$  months but did not have any young. The nursing periods were rather long. A litter of five young, still with the mother, at 56 days weighed 215 gm., and were in fair condition.

*Chart 7.*—Lot 2378 had a diet containing 9 per cent of protein. 6 per cent was derived from wheat and 3 per cent from soy beans. The growth curves were normal, showing that there is an excellent supplementary relation between the proteins of these two seeds. This illustrates, when compared with other records of animals fed 9 per cent of proteins from various sources, many examples of which are shown in these charts, the great physiological advantage







to be derived from making appropriate combinations of natural foods so as to obtain protein mixtures having high biological values.

Two females had thirty-eight young (six litters) but destroyed them all soon after birth.

Lot 2373 derived its protein supply (9 per cent) from barley and peas. The barley supplied 6 per cent and the peas 3 per cent. These animals did not grow normally and were inferior in appearance, neglectful of their young, and were old looking at 17 months.

Three females had twenty-one young (six litters) and weaned but four. Two females of the second generation were put with the family group but were killed by the others.

Lot 2371 was fed 9 per cent of protein, 6 per cent from rye and 3 per cent from peas. The animals remained in a decidedly undersized condition and had very low fertility. One female was sterile. Another had one litter of young but destroyed them soon after birth.

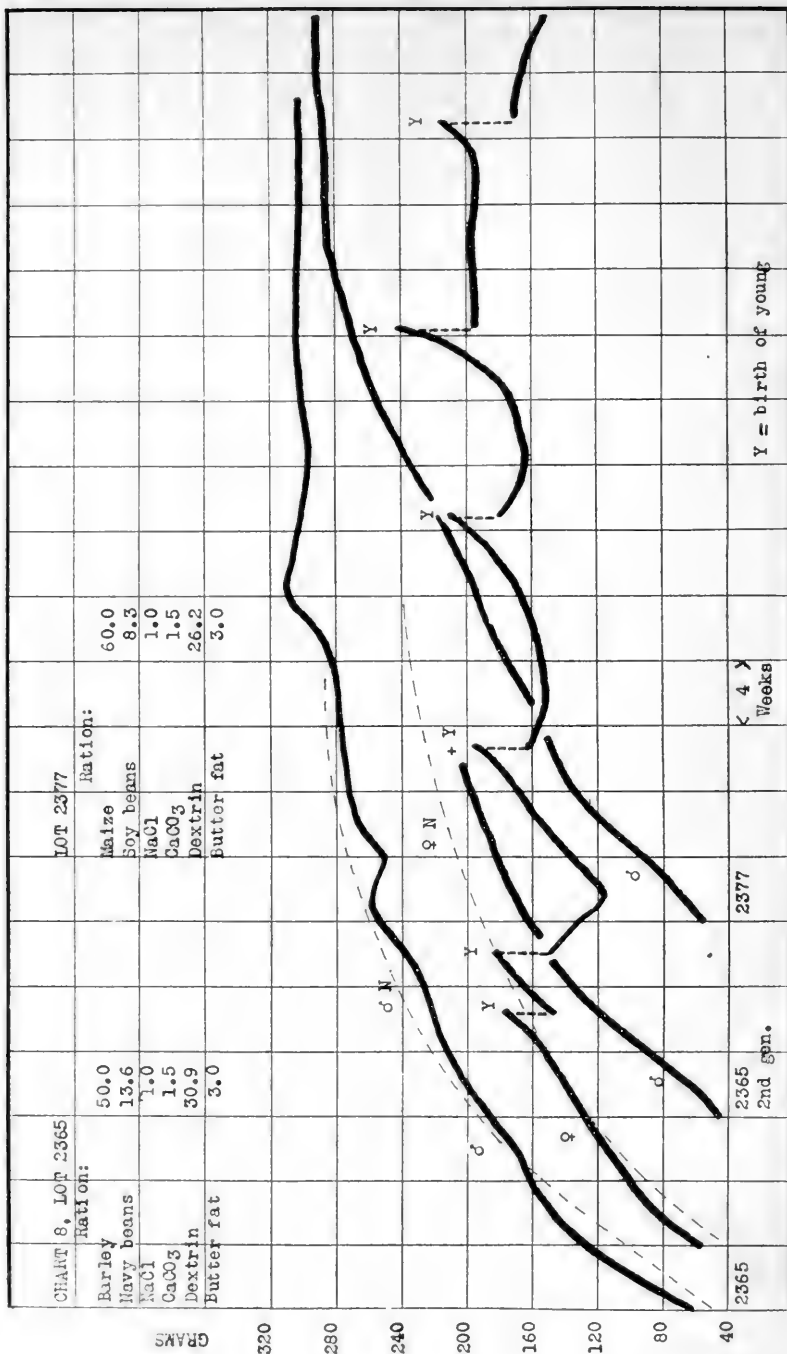
*Chart 8.*—Lot 2365 derived its 9 per cent of protein from barley (6 per cent) and navy beans (3 per cent). The growth curves were good but the animals never reached the maximum size, nor were they in first class condition. They were very senile at 18 months of age.

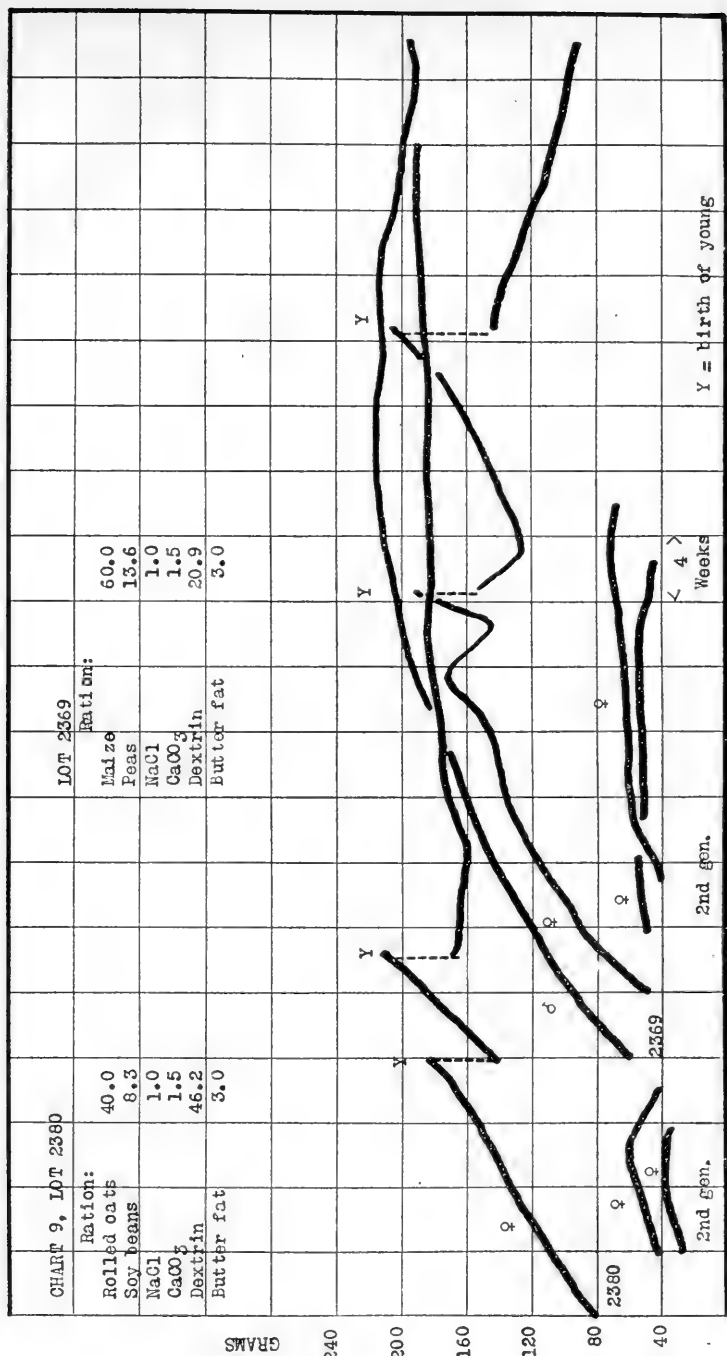
Two females had thirty-three young (seven litters) and weaned eighteen, but the young were runty. They were always small for their ages and the nursing period was long. A litter of six weighed but 75 gm. when 24 days old. At 39 days five of these weighed 118 gm. At 59 days the five weighed 168 gm. This is less than one-third what they should have weighed.

Lot 2377 was fed maize equivalent to 6 per cent and soy beans equivalent to 3 per cent of the protein. The growth curves were better than can be secured with 9 per cent of maize proteins alone or with a similar amount of soy bean protein.

The females in this group were sterile. One died at 12 months, and the other at 14 months. Both were very old looking at these ages.

*Chart 9.*—Lot 2380 derived its protein, which constituted 9 per cent of the diet, from rolled oats (6 per cent) and soy beans (3 per cent). The growth curves do not indicate any appreciable supplementary relationship between the proteins of these two seeds.





Three females were confined to this diet. One never had any young. The other two had collectively fourteen young (three litters) and weaned three, which were always undersized and puny. A litter of seven was reduced by the mother to four at 16 days. At this age the four weighed 45 gm. At 35 days of age the four weighed 105 gm. and were not in a satisfactory condition. At 51 days but three survived. These weighed 102 gm. or less than half the size normal for young of this age.

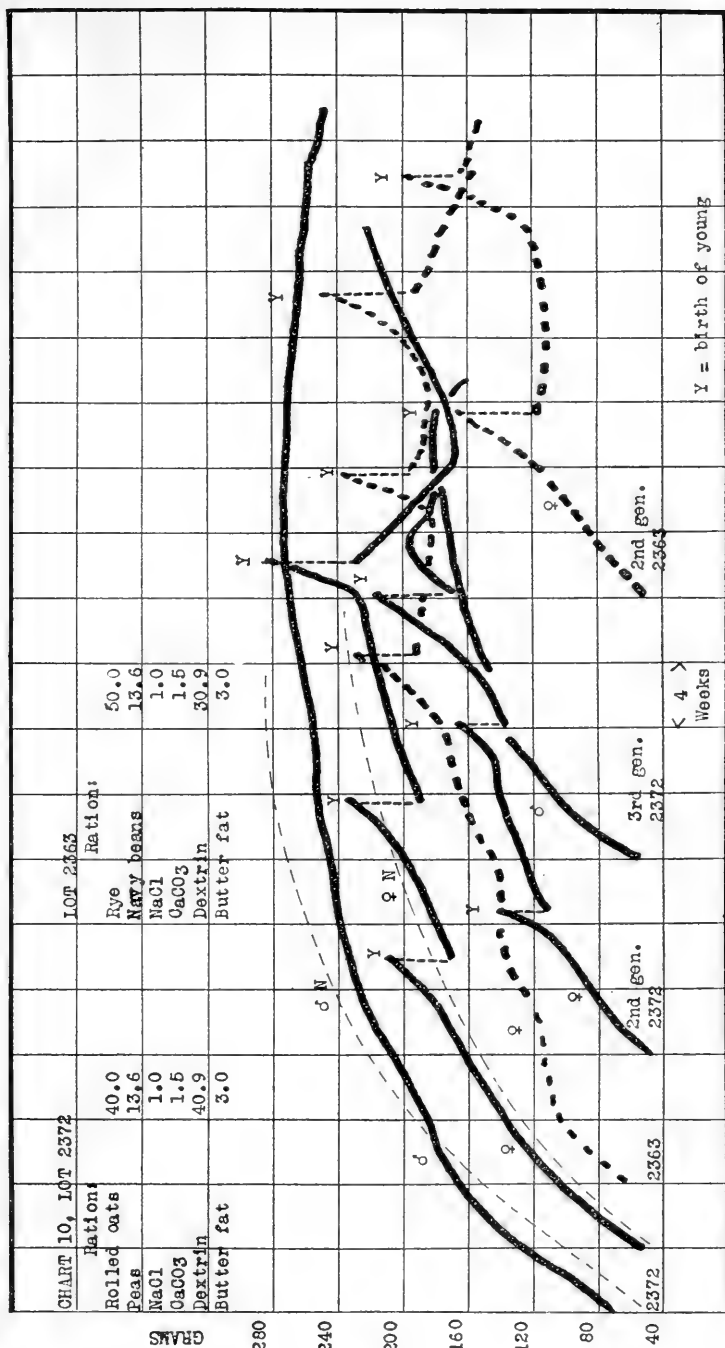
Two second generation females were confined to the diet but never grew more than a few grams and died in 2 to 3 months after weaning.

Lot 2369 was fed 9 per cent of protein, two-thirds of which was furnished by maize and one-third by peas. Growth was distinctly below normal and was very inferior to a similar combination of wheat and peas (Chart 13, Lot 2370). The animals were always undersized and were old looking at 10 months and very ragged looking at 12 months.

Two females grew up on the diet. One remained sterile. The other had fourteen young (two litters) and weaned ten. The young were runty, and required a long nursing period. A litter of six young was reduced by the mother in 7 days to four. These weighed at this time 37 gm. At 31 days the four weighed 107 gm. At 46 days the four weighed 123 gm. Two females in the second generation remained undersized and never had any young.

*Chart 10.*—Lot 2372 derived its 9 per cent of protein from rolled oats (6 per cent) and peas (3 per cent). The growth curves were somewhat below the maximum, but much better than could be secured from 9 per cent of protein from oats or peas alone. There is, therefore, an excellent supplementary relationship between the proteins of the oat and pea, but this is not so effective as is a combination of proteins of wheat and peas (Chart 13, Lot 2370). Oat and pea proteins are better than maize and peas, as is shown by a comparison with Lot 2380 (Chart 9).

There were two females in this group. One had twenty-five young (three litters) and weaned thirteen. The young were never vigorous. One female died at 6 months from an unknown cause. One second generation female had ten young (three litters) but destroyed them soon after birth. The nursing periods were long



in all cases. A litter of eight weighed 65 gm. at 14 days. At 22 days the mother had reduced the number to six, which weighed 55 gm. At 48 days there were but five remaining. These weighed 111 gm. At 76 days but three remained. These weighed 107 gm.

Lot 2363 was fed 9 per cent of protein, 6 per cent being derived from rye and 3 per cent from navy beans. The growth records and fertility were decidedly below normal. The nursing periods were greatly prolonged and the infant mortality was high. After being restricted to the diet for 18 months the animals looked very old.

Two females had collectively thirty-one young (five litters) and weaned twenty-two. A litter of nine young was reduced by the mother to eight by the 11th day. These weighed 59 gm. They were reduced to five by 34 days, and the litter weighed 80 gm. At 55 days the litter was reduced to three, weighing 85 gm. This is less than one-third the normal weight for this age. Two second generation females had twenty-three young (four litters). One third generation female had a single litter of two, but destroyed them soon after birth.

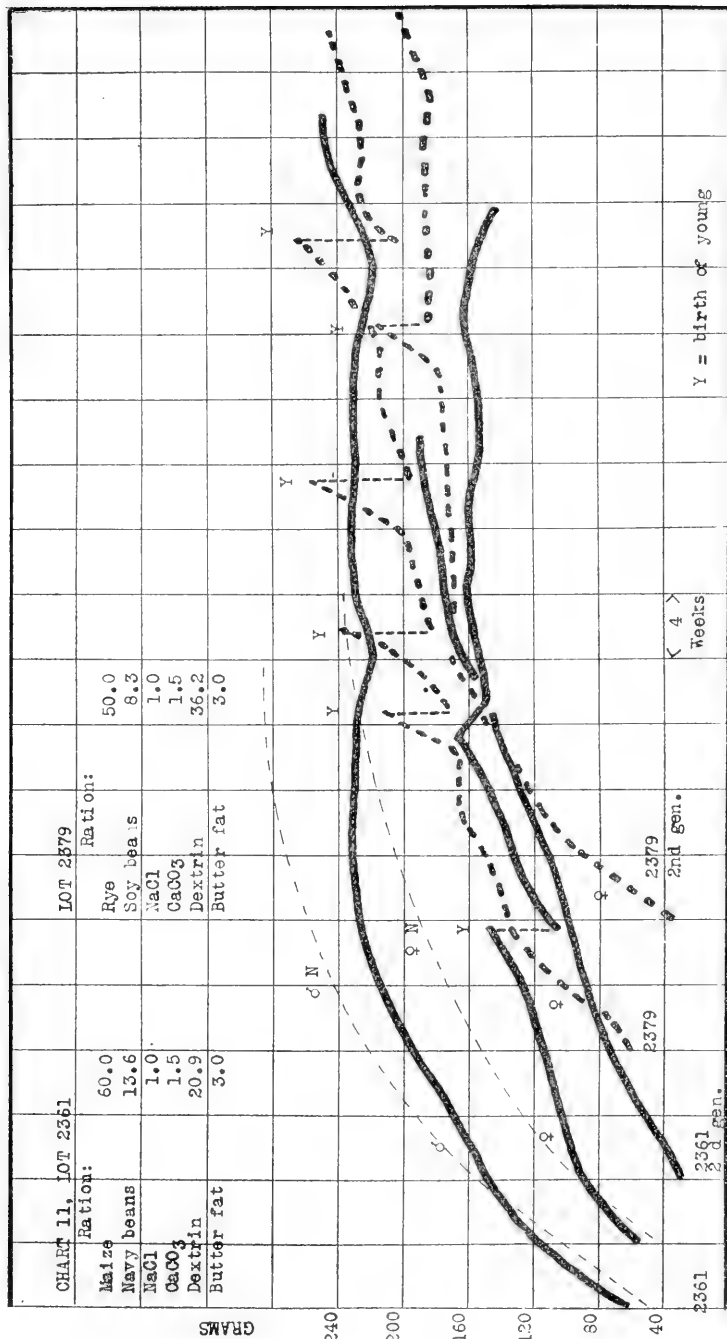
*Chart 11.*—Lot 2361 was fed maize and navy beans to furnish 9 per cent of protein in the diet. Maize furnished 6 per cent and navy beans 3 per cent of the protein. The animals grew slowly and remained somewhat undersized, their fertility was low, and infant mortality high. In Lot 814 we<sup>7</sup> have secured much better growth and reproduction on a diet containing 7.4 per cent of maize and 4.4 per cent of navy bean proteins, respectively. This is a remarkable illustration of the physiological effects which may result from a small variation in the amount of protein in the diet.

There were two females in this group (Lot 2361). They had fourteen young (three litters) and weaned eight. The young were small for their ages. One litter of seven was reduced by the mother to six at 17 days. These weighed but 60 gm. At 42 days the six weighed 145 gm. One second generation female was kept on the diet 11½ months but never had any young.

Lot 2379 derived the 9 per cent of protein in its diet from rye (6 per cent) and soy beans (3 per cent). The animals remained somewhat undersized but appeared to be in good condition.

Two females had forty-three young (five litters) and weaned fifteen. The young were never in good condition and the nursing

<sup>7</sup> McCollum, E. V., and Simmonds, N., *J. Biol. Chem.*, 1917, xxxii, 54.



period was long. Three second generation females grew up on the diet. Two of these remained sterile. The other had a litter of four and weaned one.

One of the females of the original group had a litter of eleven. At 8 days these weighed collectively 85 gm. By 20 days they were reduced by the mother to ten. These weighed 110 gm. On the 35th day five of these died. On the 41st day but two were left. These weighed but 37 gm. This was less than one-third what they should have weighed. These two remaining ones were killed and eaten by the mother at 51 days. The history of this litter of young is typical of many we have seen in groups of animals restricted to diets which were satisfactory in every respect except in quality or amount of protein.

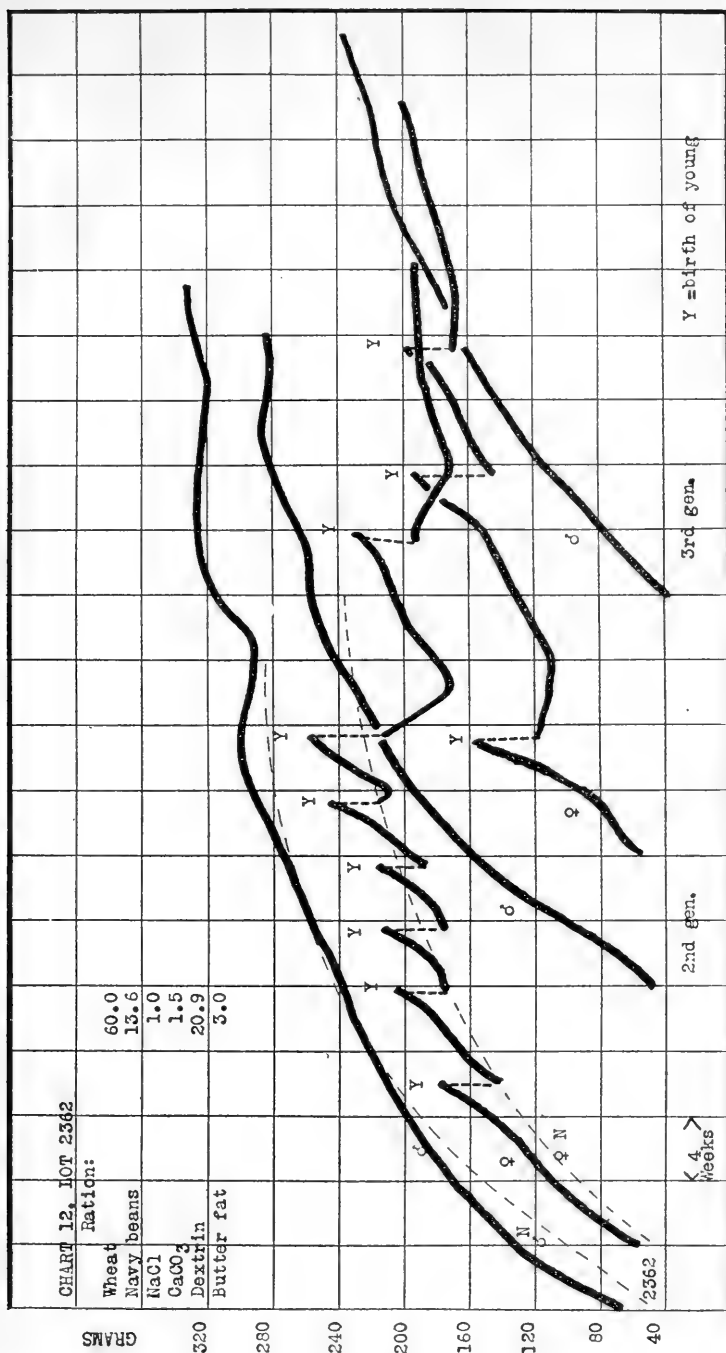
*Chart 12.*—Lot 2362 took a diet containing 9 per cent of protein derived from wheat (6 per cent) and navy beans (3 per cent). The growth records and fertility were good but the infant mortality was high.

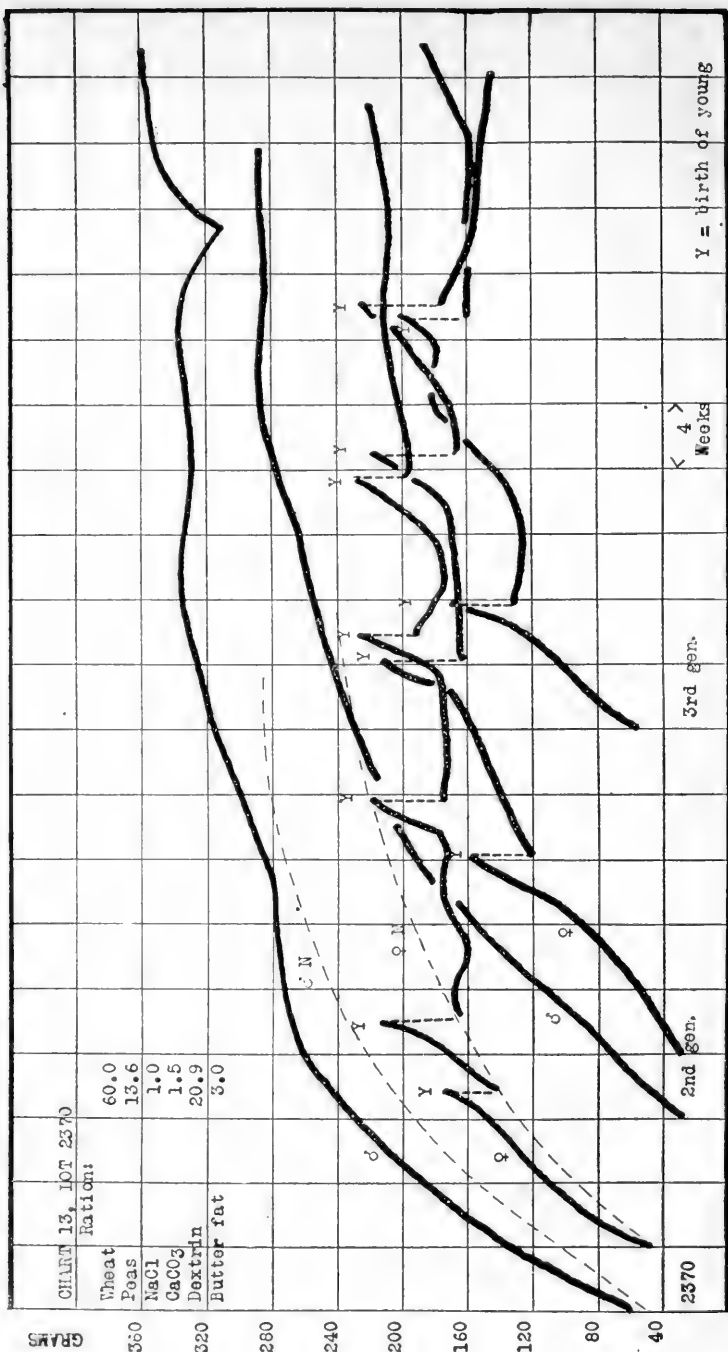
Two females had thirty-seven young (nine litters) and weaned eleven. Many young were destroyed by the mothers soon after birth. Two second generation females had thirty-five young (nine litters) and weaned seventeen. One second generation female remained sterile. The young were always inferior in size, appearance, and vigor.

*Chart 13.*—Lot 2370 derived its 9 per cent of protein from wheat (6 per cent) and peas (3 per cent). The growth curves of this group were the best we have observed in rats fed but 9 per cent of protein derived from a combination of vegetable proteins from two sources. The animals reached full adult size, the fertility was high, and the success in rearing young was better than in any other group described in this series, or in any instance in our experience where rats were grown and maintained on a diet containing but 9 per cent of *vegetable* proteins.

Two females had forty-nine young (nine litters) and weaned thirty-four. Two second generation females were grown on the family diet. One remained sterile. The other had twenty-six young (four litters) and weaned twenty-one. One third generation female had ten young (two litters) and weaned eight. The young were always somewhat small for their ages and the nursing periods rather prolonged because of the limitation placed upon the mothers by the low protein content of the diet.







## SUPPLEMENTARY PROTEIN VALUES IN FOODS.

### V. SUPPLEMENTARY RELATIONS OF THE PROTEINS OF MILK FOR THOSE OF CEREALS AND OF MILK FOR THOSE OF LEGUME SEEDS.

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(Received for publication, March 21, 1921.)

The nature of the dietary deficiencies of the cereal and legume seeds is now well understood.<sup>1</sup> It has also been shown that milk and the leafy vegetables occupy a unique place among our ordinary foodstuffs in that they are the only foods regularly consumed in moderate quantities which are of a nature to correct the mineral deficiencies of cereals, legume seeds, tubers, and fleshy roots, or to adequately supplement them with respect to fat-soluble A. It is well known from laboratory experiments on animals as well as from agricultural experience, that milk proteins tend to enhance the value of vegetable proteins generally.<sup>2</sup> Specific information as to the extent to which milk proteins supplement those of individual vegetable foods is still wanting. The studies reported in this paper form a contribution to this phase of our knowledge of practical dietetics.

In these experiments the proteins of the diets were derived from half skimmed milk powder (Merrell-Soule) and a single plant seed or tuber. In order to bring out more clearly the extent of the supplementary relations between the proteins employed we have in all cases limited the content of this factor to 9 per cent of the food mixture. We have already pointed out that in order to secure a normal growth curve in the rat on this low plane of protein

<sup>1</sup> McCollum, E. V., *The newer knowledge of nutrition*, New York, 1918.

<sup>2</sup> McCollum, E. V., *J. Biol. Chem.*, 1914, xix, 323.

intake, the quality of protein for growth must be good.<sup>3</sup> When the growth curves fall below normal the extent of retardation of development serves as a good index to the extent to which the quality of any mixture under investigation falls below that of the best combinations which we have been able to discover. When, in addition, we observe the animals throughout their reproductive period and secure records of fertility and infant mortality, and the time at which the first signs of old age appear, we have the most sensitive indexes to physiological well being which it seems probable will ever be observable. Proteins of good quality will induce normal growth when fed at the plane of intake of 9 per cent of the food mixture, and may induce a fair degree of fertility. In order to secure high fertility and low infant mortality the proteins must be of excellent quality.

In a former paper<sup>4</sup> we have described comparable studies with diets in which the proteins were derived from combinations of either liver, kidney, or muscle with cereal or legume seed proteins. The plane of protein intake was uniform (9 per cent) in all cases. These records, together with those presented in the present paper, form, therefore, a contrast between the value of milk proteins on the one hand and animal tissue proteins on the other, as supplements for a number of vegetable foods with respect to the protein moiety.

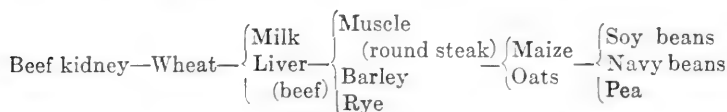
The results bring out in a very striking way the unexpected superiority of animal tissue proteins for the special purpose of enhancing the value of various plant seed proteins. In our numerous studies of this phase of nutrition we have demonstrated that with diets of the type containing 9 per cent of protein, and with all other factors satisfactorily adjusted, the proteins of kidney produce the best results we have yet observed. The biological value of the proteins of animal tissues for growth or for maintenance of health differs in an easily demonstrable degree.

An inspection of the results of our experiments with diets of the type here employed warrants arranging the proteins of a number of animal and vegetable foods of great importance in a series show-

<sup>3</sup> McCollum, E. V., Simmonds, N., and Parsons, H. T., *J. Biol. Chem.*, 1919, xxxvii, 155.

<sup>4</sup> McCollum, E. V., Simmonds, N., and Parsons, H. T., *J. Biol. Chem.*, 1921, xlvii, 139.

ing their relative nutritive values. In the following scheme we have arranged the several foods in order of the biological value of their proteins. The best is placed at the left and the series is a descending one.



In our earlier studies of the cereal grains<sup>5</sup> we became convinced that wheat proteins were of somewhat lower biological value than more recent experimental data would seem to indicate. This may perhaps be accounted for by differences in the proteins of various samples of wheat. It is well known that the dough-forming quality varies markedly in wheats. This property depends upon the peculiar nature of the proteins of this grain and may be due to lack of uniformity in the relative amounts of the individual proteins contained in the seed.

It should be kept in mind that such a differentiation in biological value of proteins from these foods will not be apparent unless the experimental procedure is appropriate to bring them out. The several foods included in the scheme must be fed, with other dietary factors satisfactorily constituted, at such a plane of intake as to furnish the critical level of 9 per cent of protein in the diet. This is the only method we have been able to devise to show these differences. The observations must include not only the period of growth but also the fertility, the success with young, and the period following the completion of growth to the point where senile characters are apparent.

We were surprised to find how consistently combinations of milk proteins and cereal or legume proteins fail to show as high biological values as can be demonstrated for kidney, liver, and muscle proteins combined with those of certain cereals.<sup>4</sup> It should not be lost sight of that milk has an effective supplementary relation to cereals both with respect to the inorganic and fat-soluble A deficiencies of the latter, whereas muscle meats supplement them

<sup>5</sup> McCollum, E. V., Simmonds, N., and Pitz, W., *J. Biol. Chem.*, 1916-17, xxviii, 211. Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1919, xxxvii, 557.

only with respect to the protein factor, and glandular organs only with respect to protein and fat-soluble A. This fact is brought out in a striking way by the records in Chart 5. When pasteurized or boiled milk, or cooked glandular organs are contained in the diet they do not effectively supplement a cereal and legume seed mixture with respect to the antiscorbutic factor since this is a labile substance. This factor is essential in the nutrition of man, monkey, and guinea pig, but need not be furnished by the diet of the rat or prairie dog, since they are apparently able to synthesize it.<sup>6</sup>

#### SUMMARY.

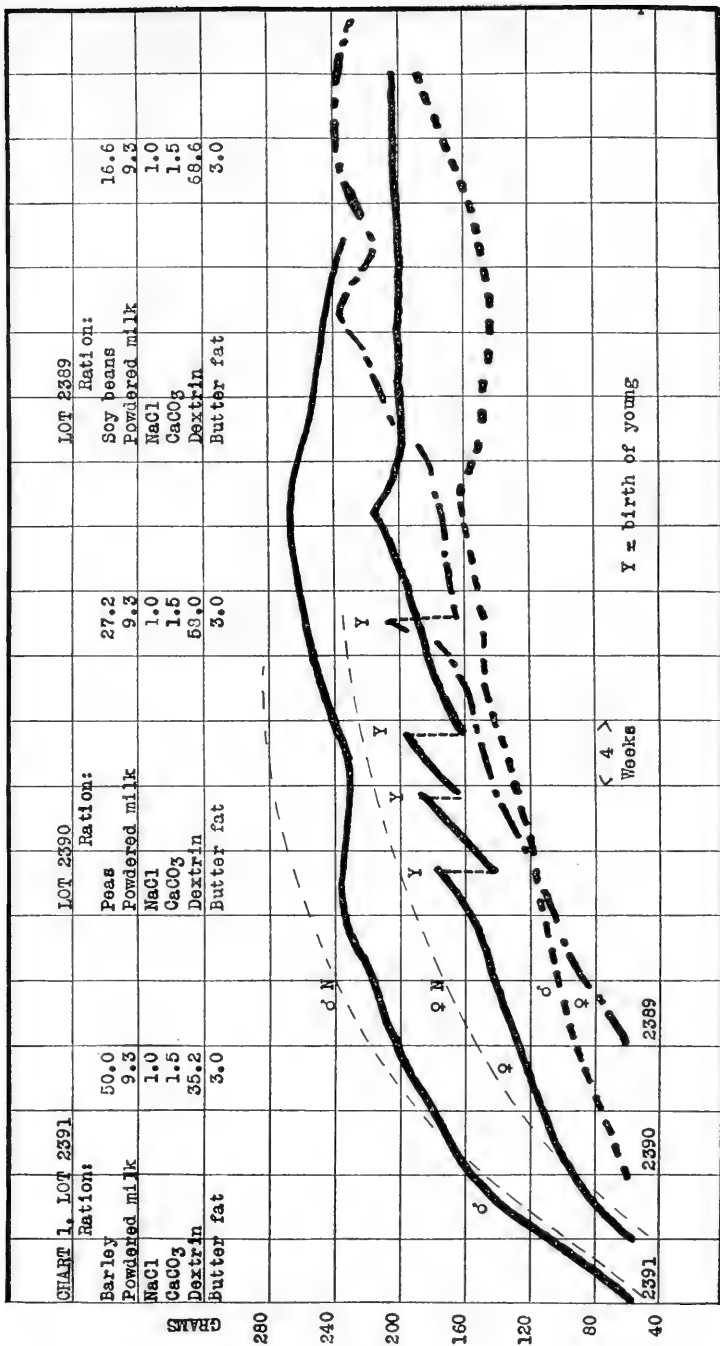
We have described in this and preceding papers experiments which were so planned as to compare the relative merits of animal tissue proteins and of milk proteins for enhancing the value of the proteins of each of the following vegetable foods: barley, peas, soy beans, rye, maize, navy beans, wheat, potato, and rolled oats. These indicate clearly that animal tissues such as liver, kidney, or muscle, are superior to milk for the specific purpose of making good the deficiencies of the proteins of the seeds and tuber mentioned above.

Milk, however, is an effective supplement for these vegetable foods with respect to other factors as well as protein. This is especially true of calcium and fat-soluble A.

In making deductions from these results it should be kept in mind that muscle tissue supplements seeds, tubers, etc., only with respect to the protein factor, and that other deficiencies of even greater importance for the well being of the body are always met with in that group of vegetable foods which are functionally storage tissues of plants; *viz.*, seeds, tubers, and roots.

*Chart 1.*—Lot 2391 was fed a diet containing 9 per cent of protein derived from barley (6 per cent) and milk (3 per cent). All other factors in the diet were made satisfactory by suitable additions of inorganic elements and fat-soluble A (in butter fat). Growth took place at a subnormal rate and the full adult size was never attained. The animals in this group looked rough coated and old at 15 months.

<sup>6</sup> McCollum, E. V., and Parsons, H. T., *J. Biol. Chem.*, 1920, xlv, 603.



Two females grew up on this diet. One remained sterile. The other had eleven young (three litters) but destroyed them soon after birth.

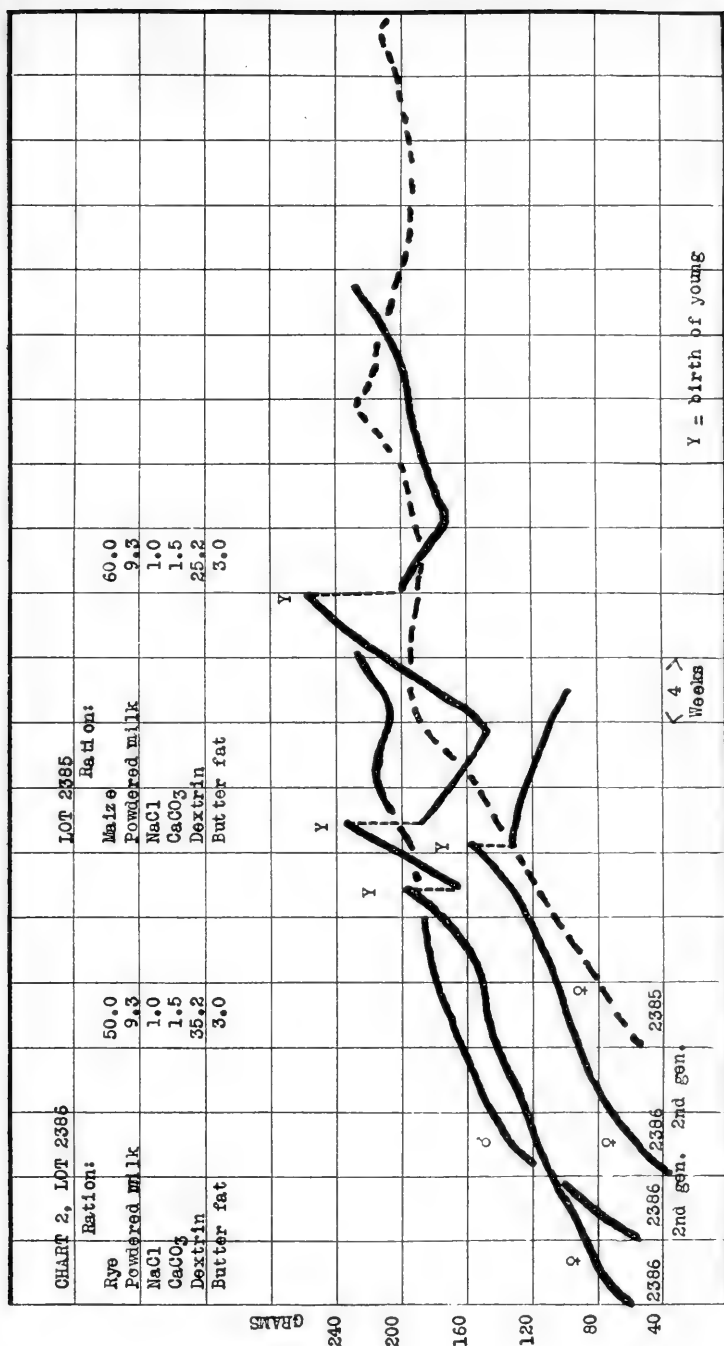
Lot 2390 derived its diet, containing 9 per cent of protein, from peas (6 per cent) and powdered milk (3 per cent). The protein of the diet was the limiting factor. The animals all grew poorly, but better than they could have grown on 9 per cent of pea protein alone. These rats were very apprehensive and could be weighed only with difficulty because of constant efforts to escape. They were so excited when handled that they would not sit still in a small covered box but would constantly spring up and strike the lid with their heads. The hair was very short and fine, and had a silky appearance, which we have never seen on rats fed highly satisfactory diets. The same type of coat has been frequently met with in rats restricted in a great measure to maize as a source of protein. Less frequently we have seen these "mole-skin" rats in groups which derived their protein from kafir corn.

Two females were restricted to this diet but never had any young.

Lot 2389 was fed soy beans and powdered milk as a source of protein. The total protein content of the diet was 9 per cent. The soy beans furnished two-thirds and the milk one-third of the total. While the growth was much better than we have ever seen on 9 per cent of soy bean protein alone, the rate of growth was distinctly below normal. The animals remained undersized, and their fertility was very low. There were three females in this group. One had a litter of seven. At 14 days they weighed 57 gm. At 21 days one had died. The remaining six weighed 62 gm. At 27 days but four survived and these weighed collectively but 45 gm. They were very puny and incapable of growing on the mother's milk. The other two females remained sterile.

*Chart 2.*—The rats of Lot 2386 were fed 9 per cent of protein derived from rye (6 per cent) and milk powder (3 per cent). Growth was slow but the animals reached nearly the adult size after some delay. These rats aged decidedly early. They looked old at 14 months. The second generation were all more undersized than the first. We have observed in many cases where the food mixture was faulty to a slight degree and a family was con-





fined to the diet through several generations, each succeeding generation was smaller than the preceding one when growth was completed.

There were three females in the group. Two of these remained sterile. The other had twenty-eight young (three litters) and weaned eleven. The nursing period was, however, very long. A litter of eight was reduced by death to five by the 32nd day. These weighed 108 gm. At 39 days they weighed 121 gm., and were in a poorly developed condition. They were less than one-third the normal size for their age. One second generation female had a single litter but destroyed it soon after birth.

Lot 2385 was fed 9 per cent of protein derived from maize (6 per cent) and milk powder (3 per cent). All other factors in the diet were made satisfactory by suitable additions. Growth was slow but the animals reached nearly the full adult size. There were three females in the group but none ever had any young. The hair of this group was short and silky and suggestive of a mole skin.

*Chart 3.*—Lot 2388 derived the 9 per cent of protein in its diet from navy beans (6 per cent) and milk powder (3 per cent). The animals grew slowly and remained permanently undersized. They lived surprisingly long on this diet on which they grew so poorly. The history of the group on 9 per cent of protein from navy beans and milk is comparable to that of Lot 2390 (Chart 1), which was identical except that peas replaced the beans. The same diet with soy beans in place of peas or navy beans produced distinctly better growth (Lot 2389, Chart 1).

Lot 2384 derived the 9 per cent protein in its diet from wheat (6 per cent) and milk powder (3 per cent). The combination of wheat and milk proteins is better than a similar amount of protein from wheat alone. With the exception of the diet of oats and milk (Lot 2387, Chart 4), this food mixture was superior to any other combination of seed with milk proteins which we have studied. The animals appeared old after about 19 months on this diet.

There were two females in the group, one of which died after being  $4\frac{1}{2}$  months on the diet. The other had forty-one young (seven litters) and weaned nineteen. The nursing periods were long in all cases. The young were not destroyed in the ruthless



manner frequently observed, but died at intervals from undetermined causes.

A litter of five young weighed but 103 gm. at 23 days of age. At 58 days four weighed 141 gm. They appeared to be in good condition but were undersized. Two second generation females had but five young (one litter) and weaned one.

*Chart 4.*—Lot 2403 derived the 9 per cent of protein which its diet contained from potatoes (6 per cent) and milk powder (3 per cent). Growth was somewhat below normal and they remained undersized. These rats aged very early. They looked as old at 1 year as many better nourished animals do at 18 months.

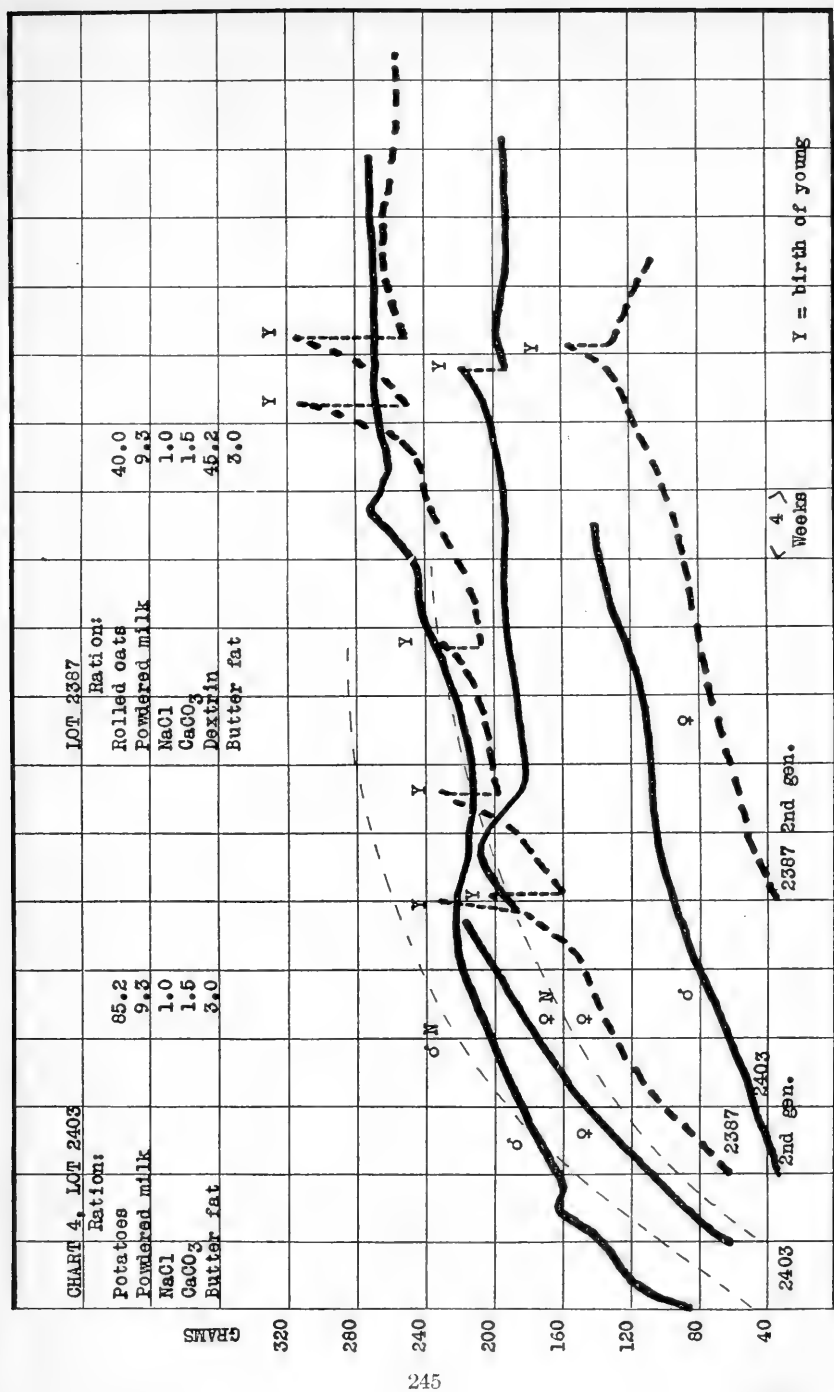
Three females had twenty-seven young (five litters) and weaned only two. The nursing periods were long. A litter of five weighed 40 gm. at 15 days. At 26 days they were reduced by death to three, which weighed collectively 47 gm. At 60 days but two were left. These weighed together 70 gm. This is less than half what they should have weighed at this age.

The nitrogen of the potato is in great measure in the form of simple substances of a non-protein nature. These substances are evidently not of a character which supplements the proteins of milk to any marked extent. We have pointed out elsewhere that the nitrogen of the potato when fed as the sole source of this factor is not of so high a value as some have reported.<sup>7</sup>

Lot 2387 was fed 9 per cent of protein, two-thirds of which was derived from rolled oats and one-third from milk powder. This combination of proteins seems to have a higher value than any other cereal and milk mixture we have investigated. But little inferior to this is the wheat and milk combination. We have in some earlier experiments seen better curves of growth on about this amount (8 per cent) of protein from a mixture of oats and milk. After 16 months on the diet their coats (Lot 2387) were somewhat rough, but the animals were still vigorous.

Three females had thirty-three young (five litters) of which six were weaned. Three other litters were destroyed before their numbers could be determined. The nursing periods were long. A litter of seven young weighed 66 gm. at 15 days of age. At 34

<sup>7</sup> McCollum, E. V., Simmonds, N., and Parsons, H. T., *J. Biol. Chem.*, 1918, xxxvi, 197.



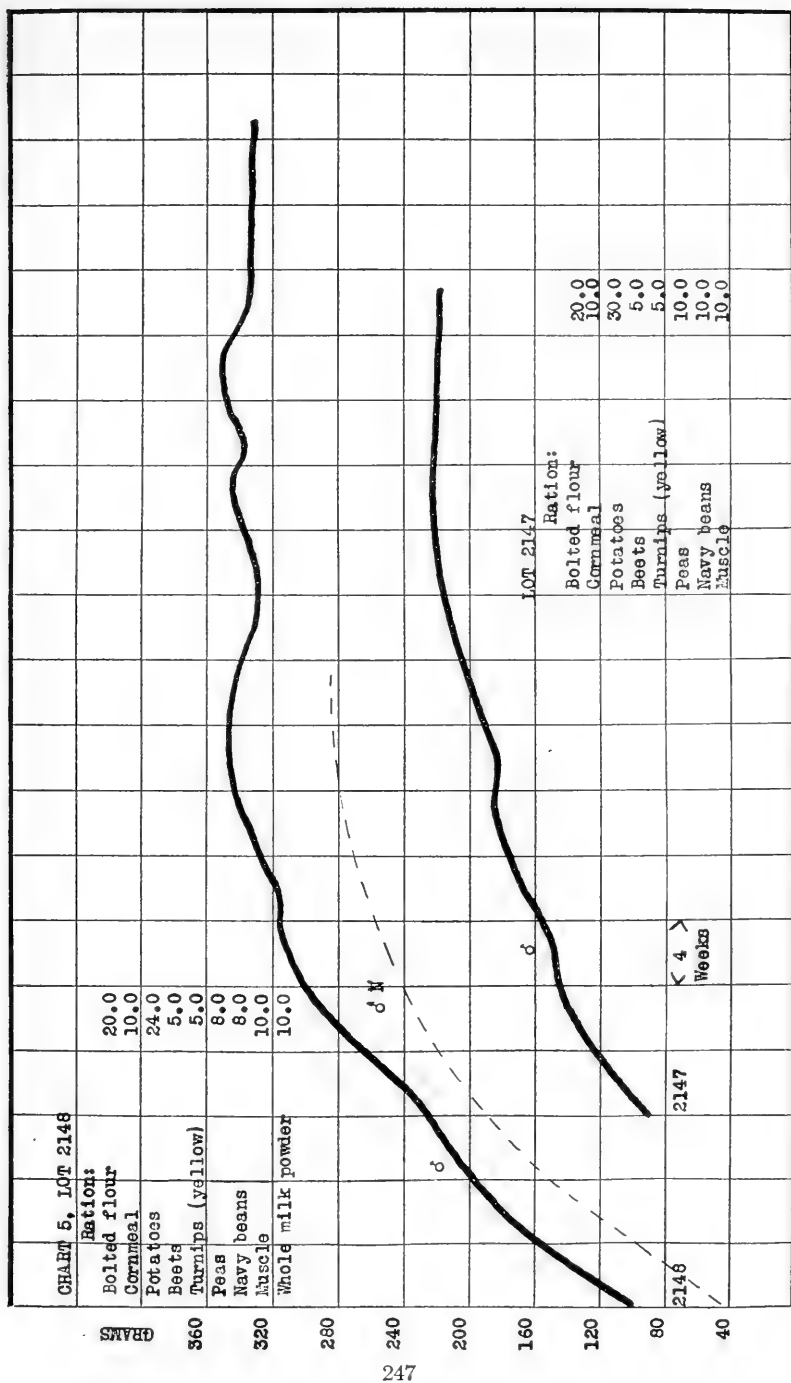
days they weighed collectively 107 gm. At 60 days six of these weighed 202 gm., or less than half the normal for this age. Two second generation females grew up on the diet. One was sterile. The other had one litter of three after she had been 8 months on the diet. She weaned them all after a long nursing period.

*Chart 5.*—Lots 2148 and 2147 show the relative merits of milk and of muscle tissue as supplements to a mixture of foodstuffs consisting of articles which are functionally storage organs of plants. The mixture, aside from the milk or muscle meat, consisted of two fleshy roots, red beet and yellow turnip; a tuber, the potato; two legume seeds, pea and navy bean; and two degerminated cereal products, wheat flour and corn-meal (maize).

Notwithstanding the wide variety in such a list of foods, and an appropriate chemical composition as indicated by the ordinary food analysis, it does not promote growth in young animals nor support the vitality of adults as measured by fertility, success in rearing young, or in deferring the onset of old age.

Muscle meat (round steak) supplements a mixture of cereals, legume seeds, fleshy roots, and tubers only with respect to the protein factor. Milk on the other hand enhances not only the protein of these vegetable foods, but likewise makes good their mineral deficiencies and also the shortage of fat-soluble A which all such mixtures exhibit.

Lot 2147 in which the vegetable diet of storage organs is supplemented only with muscle meat, failed to grow normally. The curve shown is typical of a group of six animals restricted to this diet. They never had any young and aged very early. They looked extremely rough coated after 6 to 8 months on the diet. Lot 2148, on the other hand, whose diet was similar in all respects but contained 10 per cent of whole milk powder, grew normally and remained in much better condition to an age of about 18 months. These animals showed fair fertility and success in rearing their young. The milk supplemented not only the proteins of vegetable origin but accomplished what was of greater importance; viz., the correction of the inorganic deficiencies and made good in great measure the lack of fat-soluble A. The bearing of such observations as these on practical human dietetics will be easily appreciated.







## STUDIES IN THE VITAMINE CONTENT.

### II. THE YEAST TEST AS A MEASURE OF VITAMINE B.

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AND RUTH JOHNSON.

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(Received for publication, March 30, 1921.)

Since Williams (1) first suggested the identity of Wildier's (2) "bios" with water-soluble B vitamine and proposed as a test for the vitamine the measurement of yeast stimulation, the procedure has undergone considerable investigation and criticism. As a result we now have a choice of several methods of applying the test which may be classified as follows: Measurement of yeast stimulation (*a*) by counting the cells; (*b*) by weighing the cells produced (1, 3); (*c*) by determination of the CO<sub>2</sub> produced (4); and (*d*) by determination of the volume of cells produced (5, 6). Of the methods presented to date the authors have found that the method devised by Funk and Dubin (5) combines quantitative accuracy with the simplest technique. In the work reported here we have applied this method.

In the field of criticism various types of questions and doubts have been advanced as to the specificity of the test and its adaptability as a quantitative instrument for vitamine measurement. The principal objections to its use may be reviewed briefly. Souza and McCollum (7) call attention to the sensitivity of yeast cells to many types of stimulation and advance data to illustrate this view as evidence against the accuracy of the test for evaluating vitamine content. More recently MacDonald and McCollum (8) have shown by a series of transplants that yeast will grow in a culture medium practically devoid of all but a trace of "bios" and suggest that this result is explicable on one of two hypotheses; *viz.*, that either yeasts do not require "bios" for

growth or that they can synthesize enough to meet their needs. Either explanation would make the test inaccurate for determining total vitamine in an extract.

Emmett and Luros (9) and Emmett and Stockholm (10) deny that the "bios" is vitamine B and present evidence to show that the stimulative factor possesses neither antineuritic power nor the power to induce growth in white rats. They grant the presence of a stimulative factor in extracts of vitamine-rich materials but imply that it is either a new vitamine or a factor of another sort.

A series of articles has recently appeared reporting work by three authors, Fulmer, Nelson, and Sherwood (11). In these articles they point out that the stimulative effects of alfalfa and wheat embryo extracts upon yeast growth are not directly proportional to the weights of the materials extracted. Funk had already called attention to this fact in his article describing his centrifugation method. Second, they present evidence tending to show that the stimulative effect of an extract or culture medium is greatly altered by varying the concentrations and kinds of mineral salts therein. In this connection they propose a formula for a culture medium (Medium F) which they believe will produce maximum stimulation to yeast growth and which will not be improved or altered by the addition of extracts of organic substances unless the latter disturb the optimum salt concentrations of the medium. In brief they imply that "bios" is a matter of salt concentration. Third, they advance the claim that the stimulative effect of alfalfa extracts upon growth of yeast cells cannot be changed by the treatment of the extract with alkali and this fact indicates that the stimulative factor cannot be vitamine B.

Should all these criticisms prove valid it would seem idle to proceed further with the development of the test. In reviewing these papers and our previous work we are not yet convinced that the time has come to reject the test as worthless. In the present paper we wish to present our accumulated data of the past 6 months which have been devoted to somewhat detailed investigations of behavior of the test under varying conditions.

Since the presentation of the Funk-Dubin method (6) at New Haven in May, 1920, we have adopted that technique. For

various reasons we have modified the reported method somewhat, but in the main it is essentially as described by the authors. Since, however, all the results of this article are based on the use of this test, it may be worth while to briefly describe our procedure.

### *Method.*

To a basal diet of 9 cc. of sterile culture medium (Nägeli solution, Medium F, etc.) in a sterile test-tube is added 1 cc. of the sterilized extract of the material to be examined. A pure culture of Fleischmann yeast is maintained on an agar slant and 24 hours before the test is to be made, a transplant is made to a fresh agar slant. (Funk and Dubin hold that brewer's yeast is better adapted to the purposes of the test since it is always bottom growing and packs more readily on centrifugating.) One standardized platinum loopful of the 24 hour yeast is used to inoculate the contents of each tube, the tube is then stoppered with cotton and incubated. In the experiments that follow, unless otherwise stated, the culture medium used by us has been the Nägeli solution. Our incubators are set for 31°C. and are so maintained that their maximum temperature variation lies between 30 and 35°C. For various reasons we have selected 72 hours as our period of incubation. At the end of the incubation period the yeasts are killed by plunging the tubes into water heated to 80°C. and the tubes are maintained at this temperature for 15 minutes. Unless the yeasts are killed they will not pack well in the subsequent centrifugation.

The contents of the tube are next transferred to a Hopkins' vaccine centrifuge tube which has a capillary tip graduated from 0.00 to 0.05 cc. with five markings. With a magnifier and a scale it is possible to read accurately to tenths of the divisions etched on the tip; *i. e.*, to thousandths of a cc. The tube and its contents are then centrifugated 20 minutes at a speed of about 2,500 revolutions per minute.

In the preparation of our extracts various methods were employed depending upon the nature of the source. We have found that boiling water will in 3 hours extract all the yeast stimulative substance from a small amount of dried material unless considerable starch is present. In that case we have resorted to extraction

with 95 per cent alcohol, filtered, evaporated to dryness on a steam bath, taken up the residue with water, filtered again, and diluted or concentrated the filtrate as desired. If much fat is present a preliminary extraction with alcohol-free ether will prepare the material for water or alcohol extraction. We have used each and all of these methods as the circumstances warranted. When extracting food materials it is customary to dry them to constant weight at 60°C. in an air bath. The filtered watery extracts used in the tests are determined colorimetrically for pH. In practice, if the hydrogen ion concentration falls between a pH of 6 and one of 7 no further treatment is applied to the extract. If the concentration is over 7 or under 6 we have neutralized with HCl to litmus and restored to the original volume by dilution or evaporation. Excess of alkali not only retards yeast growth but also tends to precipitate the calcium phosphates from the culture medium. Once prepared, the extracts are placed in cotton stoppered Erlenmeyer flasks and sterilized in an Arnold steam sterilizer. Two 30 minute treatments with a 24 hour interval are usually sufficient to secure sterility and once sterilized the flasks and contents can be kept indefinitely.

### I.

Our first experiments aimed to apply the above technique to a series of materials whose relative vitamine B content had been already established by rat-feeding tests. We, therefore, selected for this purpose the materials described by Osborne and Mendel (12) in a previous article in this Journal. Our tests with these sources fall into three series. In our first series we obtained fresh materials of the kind described by Osborne and Mendel, dried them at 60°, and made our extracts by boiling the dried materials with water. These preparations are those listed on the chart as "Dr. Heft's preparations." To make sure that our extraction was complete we reextracted the residues until tests showed that the stimulative factors were reduced to the limits of the controls. Since water was used in the experiments it does not follow that we obtained all the vitamine that a rat gets out in digestion, but with that reservation, we feel sure that our tested extracts contained all of the vitamine that ~~it~~ was ex-

tractable by water. The final extracts were combined and concentrated to a volume such that 50 cc. contained the extractable

TABLE I.

A comparison of the vitamine values of a series of materials as determined by Osborne and Mendel (12) in a rat-feeding experiment with the values given by the yeast test applied to water extracts of similar materials (Dr. Heft's preparations).

## The Osborne and Mendel results.

Materials used.	Gain of weight of rats fed 1 gm. daily.				Total.	Average.			Gain of weight of rats fed 0.5 gm. daily.			Total.	Average.	
	1	2	3	4					1	2	3			
Alfalfa.....	200	179	146		525	175	1		144	121	106	371	124	1
Clover.....	197	177	150		524	174	2		112	102	99	313	99	2
Tomato.....	122	120	118	115	475	119	3		88	70		158	79	3
Spinach.....	119	101	81	75	376	94	4		87	61	32	180	60	4
Cabbage.....	99	91	58		248	82	5		52	49	30	131	43	5
Turnip.....	96	68	66		230	77	6		46	30	20	96	32	7
Carrot.....	78	78	66	46	268	67	7		49	38	36	123	41	6
Timothy.....	42	40	10		92	31	8		22	5	—2	25	8	8
Beet.....	6	—9	—3		—6	—2	9		No data on 0.5 gm.					

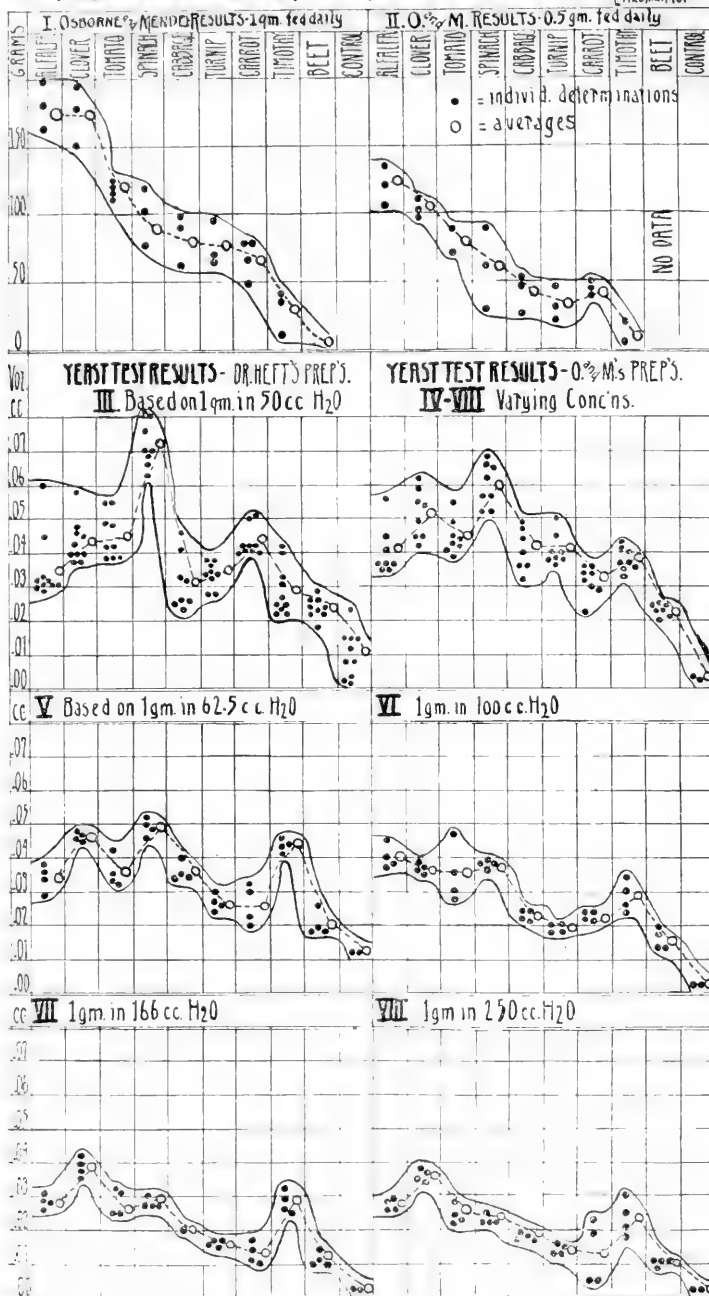
The yeast test results. 1 cc. of water extract contained the extractable "bios" of 0.02 gm. dried material. 1 cc. of extract and 9 cc. of Nägeli solution were used in each determination.

Materials in Osborne and Mendel order of value.	Individual determinations in thousandths of a cc. of yeast cells.										Total.	Average.	Rating.
	1	2	3	4	5	6	7	8	9	10			
Alfalfa.....	60	45	33	32	32	31	31	31	30	29	354	35	5
Clover.....	58	48	46	45	43	40	40	40	38	38	436	44	3
Tomato.....	55	55	49	48	42	42	39	39	39		408	45	2
Spinach.....	82	31	76	70	70	69	66	63			577	72	1
Cabbage.....	46	41	33	32	26	26	25	23			252	31	7
Turnip.....	38	38	37	35	34	34	33	28	28		305	34	6
Carrot.....	51	50	42	42	42	41	41	40	40		389	43	4
Timothy.....	42	40	33	31	27	25	25	23	22	22	290	29	8
Beet.....	29	26	26	24	24	23	22	22	18		214	24	9
Control.....	23	15	15	15	12	8	8	23	2	1	122	12	

material from 1 gm. of dried material. Table I shows the results obtained with the materials of the first series and Chart I is a graphic presentation of the same.

CHART I.

## A COMPARISON OF FEEDING TESTS AND THE YEAST VITAMINE TEST

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While we may justifiably draw from these results an approximate agreement with the Osborne-Mendel conclusions the agreements are not exact and in the case of spinach there is a very marked discrepancy. We felt at the time that our variations might quite possibly be due to variability in the vitamine content of our materials as compared with the Osborne and Mendel

TABLE II.

Results of the yeast test applied to varying concentrations of water extracts of the actual materials used in the Osborne and Mendel experiments.\*

A. 1 cc. of extract tested carried the extractable "bios" of 0.02 gm. dried material. 1 cc. extract plus 9 cc. Nägeli solution used in each test.

Materials in Osborne and Mendel order of value.	Readings in thousandths of a cc.								Total.	Aver- age.	Order of value.
	1	2	3	4	5	6	7	8			
Alfalfa.....	56	45	41	37	37	37	36	36	325	41	4
Clover.....	62	59	55	54	49	45	45	42	411	51	2
Tomato.....	55	49	45	44	43	42	41	39	358	45	3
Spinach.....	68	66	65	62	57	57	52	52	479	59	1
Cabbage.....	49	47	44	40	36	36	32		284	40	6
Turnip.....	50	45	40	40	39	38	38	37	327	41	5
Carrot.....	36	36	34	34	32	30	29	22	253	31	8
Timothy.....	43	41	40	37	37	36	35	33	302	37	7
Beet.....	25	24	24	23	23	21	20		160	22	9
Control.....	2	3							5	2.5	10

B. Concentration: 1 cc. carries "bios"  
of 0.016 gm. dried extract.

C. Concentration: 1 cc.  
carries 0.01 gm.

Materials.	1	2	3	4	Total.	Average.	Order.	1	2	3	4	Total.	Average.	Order.
Alfalfa.....	38	36	34	29	137	34	6	37	38	40	45	160	40	1
Clover.....	45	46	47	48	186	46	2	35	36	37	38	146	36	3
Tomato.....	32	33	35	42	142	35	5	28	30	35	47	140	35	4
Spinach.....	46	49	50	52	197	49	1	36	38	38	39	151	37	2
Cabbage.....	34	34	35	40	143	36	4	21	22	24	24	91	22	6
Turnip.....	24	26	27	30	107	27	7	18	18	20	20	76	19	8
Carrot.....	20	22	30	32	104	26	8	21	22	24	24	91	22	7
Timothy.....	41	42	43	46	172	43	3	23	26	30	34	113	28	5
Beet.....	18	18	19	26	81	20	9	13	13	17	19	62	15	9
Control.....	2	2			4	2	10	2	2			4	2	10

TABLE II—*Concluded.*

D. Concentration: 1 cc. carries "bios" of 0.006 gm. dried material.								E. Concentration: 1 cc. carries 0.002 gm.							
Materials.	1	2	3	4	Total.	Average.	Order.	1	2	3	4	Total.	Average.	Order.	
Alfalfa.....	26	28	29	31	114	28	2	26	26	28	29	109	27	2	
Clover.....	36	38	40	42	156	39	1	34	35	37	38	144	36	1	
Tomato.....	25	25	31	32	113	28	3	22	23	24	28	97	24	3	
Spinach.....	27	27	27	30	111	27	5	22	22	22	25	91	22	5	
Cabbage.....	20				20	20	6	17	17	18	19	71	18	6	
Turnip.....	15	16	17	17	65	16	7	13	13	15	16	57	14	7	
Carrot.....	10	11	14	17	52	13	8	5	5	19	23	52	13	8	
Timothy.....	25	26	29	32	112	28	4	18	21	25	30	94	23	4	
Beet.....	10	10	11	14	45	11	9	9	10	10	10	39	9	9	
Control.....	2	2			4	2	10	2	2			4	2	10	

\* For graphic presentation see Chart I.

sources. To check this we sought a test upon the actual materials used in the Osborne and Mendel experiments and these materials were kindly furnished us for the purpose by Professor Mendel. With the actual materials at hand we repeated our tests and in this set of experiments we varied our procedure by testing various concentrations of each extract. Table II gives the actual results and these are also reproduced graphically in Chart I.

The most significant features of these results are the extreme variability in individual determinations in the higher concentrations, lessened variability, and closer approximation to the feeding test results when diluted extracts are used. The behavior of the diluted concentrations is we think more than a coincidence. Funk (5) and others (11) have shown that the curve of stimulation under varying concentrations approximates the logarithmic curve and that to get sharp contrasts between two extracts it is necessary to test dilutions which will fall on the steep part of the curve. This point is emphasized in the next series of experiments.

## II.

The results with the varying concentrations suggested that we proceed next to the establishment of the curve of reaction of a single material. To this end we first selected dried alfalfa meal



as our material and, keeping all factors constant except concentration, proceeded to determine the stimulative activity in each concentration. To obtain our extract, 400 gm. of dried alfalfa meal were repeatedly extracted with boiling distilled water, the filtered extracts combined, refiltered, and concentrated to a degree that 1,000 cc. contained the extractable material from 400 gm. In working with this extract we were at first bothered by the tendency to sedimentation on standing or centrifugating. To avoid this complication we employed a procedure described by Osborne and Wakeman for removing protein from spinach extracts. This consisted in diluting the water extract with 95 per cent alcohol to 40 per cent of the volume and filtering off the precipitated protein complex. This procedure is attended with variable results. Sometimes the precipitate carries down with it all the stimulative substance in the extract. At other times very little loss results. In the extract which we used tests showed that the loss was less than 0.002 cc. expressed in terms of yeast tests made before and after treatment. The alcohol treatment naturally diluted our extract and to remove the alcohol and restore the desired concentration the filtrate was concentrated on the water bath to the original 1,000 cc. The tests reported below were made on this material and variations in concentration are expressed as fractions of the maximum concentration. Table III and Chart II show six series of tests. In Series A, B, D, and E each determination was made by combining 1 cc. of the alfalfa extract with 9 cc. of Nægeli solution. In Series C, Fulmer, Nelson, and Sherwood's Medium F was substituted for the Nægeli solution. Series F was obtained with a filtered autolysate of Fleischmann yeast cakes. One pound of the moist cakes was mixed with water and allowed to autolyze. The mixture was then filtered and concentrated to such a volume that the highest concentration used represents the extractable material from 3 gm. of Fleischmann yeast cake in each cc. of extract.

The general shape of the curves of Series A and B is the same, the only difference being in the height of Series B and in the lower control value with this extract, possibly due to repeated heating in the 2 months that elapsed between the two tests. Both show a steep ascent to an optimum with little variability to the left

of the optimum. On the right of the optimum there is not only much wider variation in the test results but a marked decline. These results confirm those of Fulmer, Nelson, and Sherwood (11). These peculiarities show the futility of attempting to compare the value of two extracts unless the curves of the two are known and the impossibility of depending upon comparisons based on weights

TABLE III.

The yeast growth curve determined by varying the concentrations of a given extract. Time of incubation 72 hours in each determination. The results are given in thousandths of a cc. of yeast cells.

Series A. Alfalfa extract plus Nægeli solution. Maximum concentration represents the extractable material from 0.4 gm. dry alfalfa meal in 1 cc. of water.

Concentration .....	1	0.9	0.8	0.7	0.6	0.5	0.45	0.4	0.35	0.3	0.25	0.2	0.15	0.1	0.05	Control.
	22	23	27	30	24	24	29	34	36	35	36	29	30	21	20	6
	23	24	28	30	25	28	31	35	38	37	36	30	34	24	22	7
	24	28	31	33	28	28	33	35	39	37	36	32	34	27	25	6
	26	29	33	35	35	29	35	37	39	37		32		27	30	7
						29		37		39		33		27		6
						31		38		40		34		31		5
						35		38		40		34		31		
						36				41		35		32		
Total.....	95	104	119	128	112	240	128	254	152	306	108	259	98	220	97	37
Average.....	24	26	29	32	28	30	32	36	38	38	36	32	32	27	24	6

Series B. Made 2 months later but with the same extract of alfalfa used in the Series A tests.

Concentration.....	0.95	0.75	0.65	0.55	0.475	0.425	0.375	0.325	0.275	0.225	0.175	0.125	0.075	0.045	0.035	0.025	0.015	Control.
	17	24	25	23	28	28	30	29	28	29	23	25	21	18	16	13	14	2
	20	26	25	25	29	29	33	33	29	29	27	26	21	18	16	15	15	2
	20	27	26	30	34	34	33	33	29	30	29	26	22	19	17	15	17	
	21	28	26	30	35	35	34	34	32	30	30	27	22	19	18	15	17	
	25	28	27	31	36	36	34	35	35	32	33	29	24	19	19	16	18	
Total.....	103	133	129	139	162	162	164	164	153	150	142	133	110	93	86	74	81	4
Average.....	20	27	26	28	32	32	33	33	31	30	28	27	22	19	17	15	16	2

TABLE III—*Continued.*

Series C. Made with the same extract of alfalfa as Series A and B but with Fulmer, Nelson, and Sherwood's Medium F in place of Nægeli solution.

Concentration....	0.95	0.75	0.65	0.55	0.475	0.425	0.375	0.325	0.275	0.225	0.175	0.125	0.075	0.045	0.035	0.025	0.015	Control.
	30	37	32	34	30	32	28	28	26	27	26	23	20	18	18	17	18	13
	37	38	32	35	30	33	29	29	30	28	26	25	22	18	19	18	19	14
	42	42	42	35	31	34	29	32	30	28	27	25	23	20	20	19	20	15
	43	55	44	46	34	34	33	33	30	30	29	26	23	20	20	19	20	16
	62	55	45	47	36	35	35	34	33	30	29	26	23	22	21	20	21	17
Total.....	214	227	195	197	161	168	154	156	149	143	137	125	111	98	98	93	98	75
Average.....	43	45	39	39	32	33	31	31	29	28	27	25	22	19	19	18	19	15

Series D and E were made with alfalfa extract and Nægeli solution at the same time as Series B in order to determine the curvature of the steep part of the alfalfa curve. A concentration of 0.1, therefore, represents the extractable material from 0.04 gm. alfalfa in 1 cc. of water.

Series D.

Concentration.....	0.1	0.09	0.08	0.07	0.06	0.05	0.04	0.03	0.02	0.01	Control.
	25	20	22	20	18	16	15	14	13	10	2
	25	22	22	21	18	16	16	15	14	10	2
	25	23	23	21	19	17	16	15	14	10	
	25	25	23	22	20	19	16	16	14	10	
	25	25	24	22	20	19	16	16	14	11	
Total.....	125	115	114	106	95	87	79	76	69	51	4
Average.....	25	23	23	21	19	17	16	15	14	10	2

Series E.

Concentration.....	0.01	0.009	0.008	0.007	0.006	0.005	0.004	0.003	0.002	0.001	Control.
	9	9	8	8	7	7	5	4	3	2	2
	9	9	8	8	7	7	6	5	4	3	2
	9	9	9	8	7	7	7	6	5	4	
	10	9	9	8	7	7	6	5	5	3	
	10	10	9	8	7	7	6	5	5	3	
Total.....	47	46	43	40	35	35	30	25	22	15	4
Average.....	9	9	8	8	7	7	6	5	4	3	2

TABLE III—*Concluded.*

Series F. Made with yeast autolysate in place of alfalfa extract. In the maximum concentration 1 cc. of autolysate contains the extractable substance from 3 gm. of Fleischmann yeast cake. In this series the autolysate was combined with Nägeli solution.

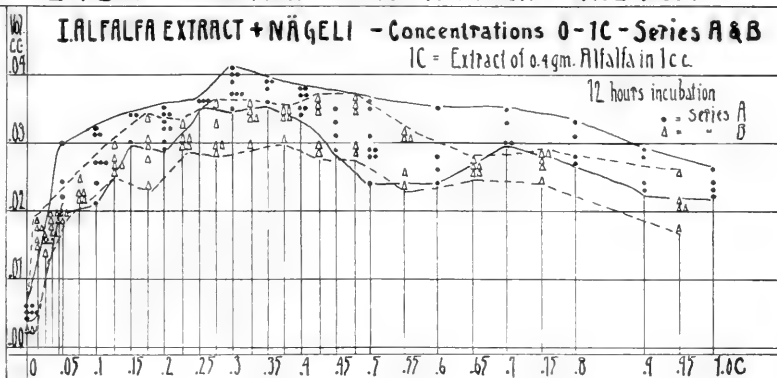
Concentration.....	1	0.61	0.56	0.48	0.4	0.32	0.24	0.2	0.16	0.12	0.1	0.09	0.08	0.07	0.05	0.04	0.03	0.02	0.01	Control.
	68	44	62	68	35	63	60	65	52	65	69	55	27	32	47	45	41	28	23	2
	68	70	63	70	36	65	68	80	60	69	70	69	36	48	47	42	39	30		2
	70			70	38	68	68		67	80			46							2
	70				52	70	70		67	80			46							2
	70				70	75	70		69				50							
							70		70				56							
Total..	346	114	125	208	231	341	406	145	385	294	139	124	261	32	95	92	83	67	53	8
Average..	69	57	62	69	46	68	67	72	64	73	69	62	43	32	47	46	41	33	26	2

of extracted materials as already pointed out by Funk and Dubin (5) and Fulmer, Nelson, and Sherwood (11). The shape of the curve also established the following data concerning the behavior of alfalfa extract. First, that no matter how concentrated the extract, it was impossible to develop a stimulative effect greater than the production of about 0.04 cc. of yeast cells in 72 hours for each cc. of extract used; second, that increasing the concentrations beyond the optimum lowers the production of yeast. The question naturally arises as to whether these features are common to all extracts; whether the decline to the right of the optimum is due to hydrogen ion effect; and whether time of incubation is a factor? The yeast autolysate curve shows that while its shape is essentially the same as that of alfalfa in showing steep rise, optimum concentration, and decline in higher concentration, its power to produce growth of yeast cells exceeds the optimum power of alfalfa extract. This fact alone would be sufficient to show that in making the yeast test we are concerned with other factors than the presence of vitamine B and its concentration. In Table IV are given the results obtained by applying the test to a variety of materials.

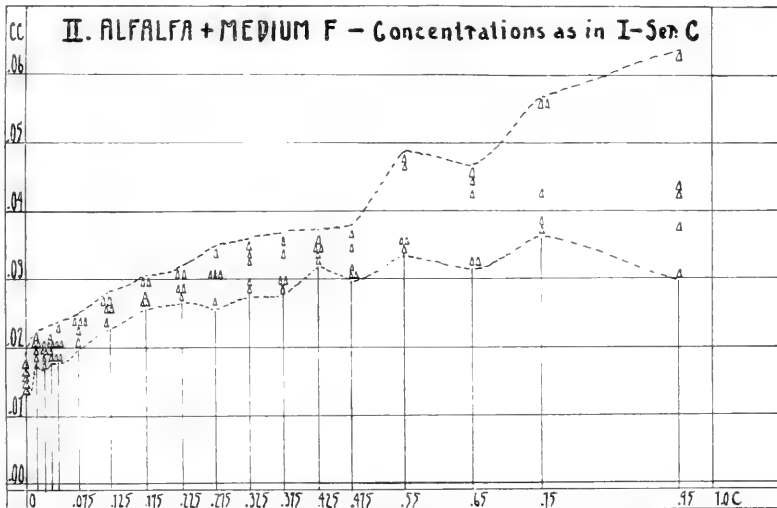
# CHART II. EFFECT OF VARIATION IN CONCENTRATION ON THE TEST

## I. ALFALFA EXTRACT + NÄGELI - Concentrations 0-1C - Series A & B

1C = Extract of 0.4gm. Alfalfa in 1cc.

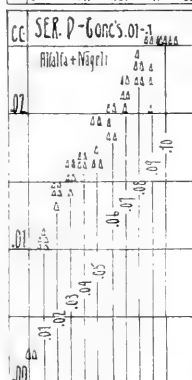


## II. ALFALFA + MEDIUM F - Concentrations as in I - Ser C



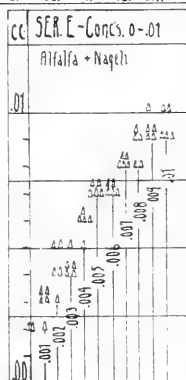
### SER. D - Conc's. 0-1

Alfalfa + Nägeli



### SER. E - Conc's. 0-01

Alfalfa + Nägeli



### SERIES F - YEAST AUTOLYSATE + NÄGELI

C = 3gm. Fleischman Yeast  
Cake in 1cc H<sub>2</sub>O

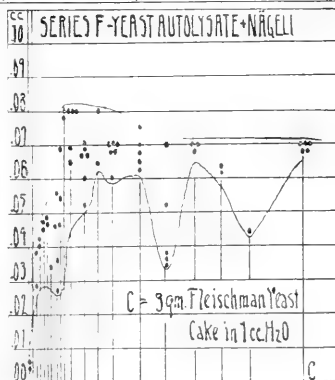


TABLE IV.

Results of applying the yeast test to varying concentrations of extracts of the materials named.

Test results with a dilute water solution of Funk's 1913 crystalline complex (vitamine). 0.0079 gm. was dissolved in 75 cc. of water. This solution strength is marked 1 in the series below and further dilutions were made for test purposes as indicated. The results are given in thousandths of a cc.

Concentration.....	1	0.8	0.5	0.4	0.3	0.2	0.1	0.08	0.05	0.03	Control.
	7	7	6	5	4	4	4	4	4	4	2
	7	7	6	6	6	4	5	4	4	4	2
	7	7	7	6	6	5	5	4	4	4	
	7	7	7	6	6	5	5	4	4	4	
	8	8	7	6	6	5	5	4	5	4	
Average.....	7	7	6	5	5	4	4	4	4	4	2

A comparison of extracts made from gland material extracted first with neutral alcohol and the residue reextracted with acidified alcohol.

Concentration.....	1. Neutral alcohol extracts original glands.					Gland.	2. Acid alcohol extracts of the residues from 1.				
	C	0.8C	0.5C	0.2C	Control.		C	0.8C	0.5C	0.2C	Control.
	20	22	23	18	5	Sheep pancreas. C equals 2 gm. fresh gland in a cc.	13	13	13	11	3
	29	23	24	20	6		13	15	14	11	2
Average.....	24	22	23	19	5		13	14	13	11	2
	15	13	9	7	5	Human pancreas.	10	9	8	5	3
	15	14	13	7	6		10	10	8	6	2
Average.....	15	13	11	7	5		10	9	8	5	2
	9	8	6	4	5	Human liver.	8	7	5	4	3
	10	8	7	4	6		8	7	7	4	2
Average.....	9	8	6	4	5		8	7	6	4	2

The only new point brought out by these tests relates to the gland extracts. Swoboda (13) in a recent article in this Journal advanced the view that Williams' results with pancreas were due to his use of acid in making his extract, since with the method employed he was unable to get stimulative effects on yeast growth with pancreatic extract. He formulated a hypothesis of vitamine existing as a vitaminogen. The tests in Table IV were designed primarily to test this view. The results fail to confirm either of his contentions. We get stimulation well above the control with a neutral alcohol extract of pancreas and the residues on reextraction with acidified alcohol yield extracts of lower potency. If the vitaminogen view were correct the latter should exceed the first in power. The concentrations used were not sufficiently great to add much data to the question of curve shape, or location of optima. A study, however, of the tests with the Osborne and Mendel materials enables us to conclude that the optima vary with the material extracted.

The decline of growth in our concentrations of alfalfa extract higher than the optimum could not be due to the pH, for its values (determined colorimetrically) in these inhibitory concentrations differed too little from that of the optimum concentration to function as an inhibitory factor in this experiment.

To answer the query as to the effect of time of incubation on our results we tested out the rate of growth of yeast cells in two concentrations of the alfalfa extract as shown in Table V and Chart III. The concentrations selected were the one found optimum in the Series B tests and the full concentration of the extract. The results show that after 24 hours and up to a period of 19 days the time factor does not change the result. The concentration found optimum in the earlier tests retains its superior stimulative potency regardless of the time of incubation.

These results do not answer all the questions raised by the alfalfa curve. They do, however, make it clear that if vitamine B is one of the factors concerned in the combined "bios" effect of an extract it is only one of several factors concerned and that the use of the yeast test for quantitative measurement of vitamine content must await the development of a medium which shall be optimum for all factors except vitamine, exactly the situation that had to be met in the development of basal diets for rat-feeding experiments.

## III.

The assumption that it is possible to develop a basal diet for yeast cells, optimum in all except vitamine B implies that at least one of the stimulative factors is vitamine B. If Fulmer,

TABLE V.

The effect of the period of incubation on the rate of growth of yeast cells in the optimum concentration of alfalfa extract as contrasted with the higher concentration of the extract.\*

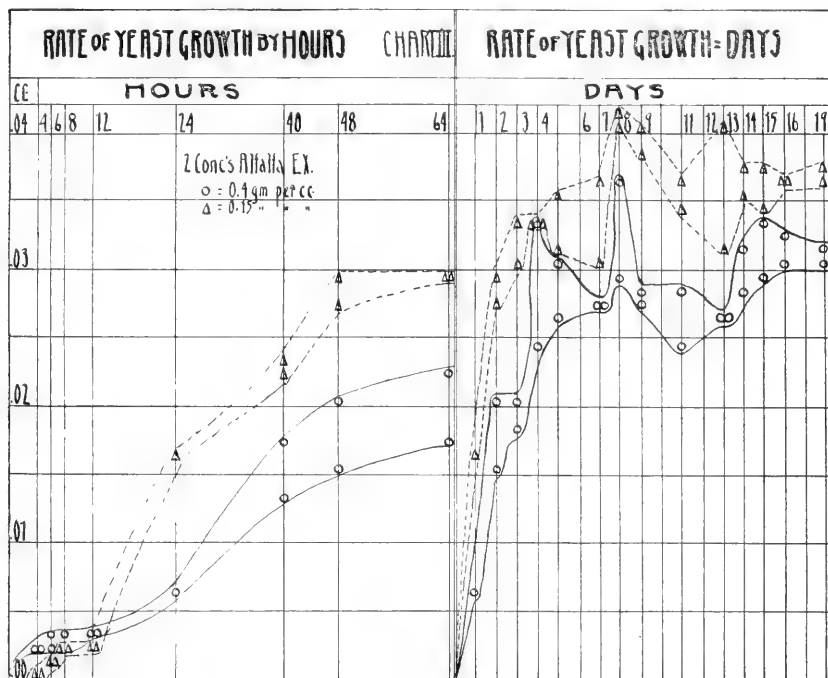
Period of incubation.	Concentration I. 1 cc. contains the extract of 0.4 gm. of alfalfa.			Concentration II (optimum). 1 cc. contains the extract of 0.15 gm. of alfalfa.		
	Test.	Test.	Average.	Test.	Test.	Average.
<i>hrs.</i>						
4	2	2	2	0	0	0
6	2	3	2.5	1	1	1
8	2	3	2.5	2	2	2
12	3	3	3	2	2	2
24	6	6	6	16	16	16
40	13	17	15	22	23	22.5
48	15	20	17.5	27	29	28
64	17	22	19.5	29	29	29
<i>days</i>						
1	6	6	6	16	16	16
2	15	20	17.5	27	29	28
3	18	20	19	30	33	31.5
4	29	33	31	38	38	38
5	26	30	28	32	35	33.5
7	27	27	27	30	36	33
8	28	36	32	40	41	40.5
9	27	26	26.5	38	40	39
11	24	28	26	34	36	35
13	26	26	25	31	40	34.5
14	28	31	27.5	35	37	36
15	29	33	31	34	37	35
16	30	32	31	36	36	36
19	30	31	30.5	36	37	36.5

\* For graphic presentation see Chart III.

Nelson, and Sherwood's contentions (11) are true even this factor is eliminated and further attention to development of the test is useless. Their contentions rest upon two different pieces of evidence; *viz.*, the claim that by proper selection of a medium



of known constituents it is possible to obtain a medium that will produce a growth of yeast cells which is not improved by the addition of organic extracts, and second that the stimulative power of extracts on yeast growth is not affected by alkali treatment of these extracts, vitamine B being known to be extremely sensitive to the destructive effect of alkali, especially when combined with heat.



To settle these points we felt that a fair test would be to substitute for the Nägeli solution the Medium F of Fulmer, Nelson, and Sherwood and see if our tests would still yield increased growth phenomena when this medium was supplemented with alfalfa extract. The differences in the two media are shown below.

Nägeli solution.	Medium F.
100 cc. distilled water.	100 cc. distilled water.
1 gm. ammonium nitrate.	0.188 gm. ammonium chloride.
0.05 " calcium phosphate.	0.10 " calcium chloride.
0.5 " $K_2HPO_4$ .	0.10 " $K_2HPO_4$ .
0.25 " magnesium sulfate.	0.04 " precipitated calcium carbonate.
10.0 " dextrose.	0.6 " dextrin.
	10.0 " sucrose.

In using the F medium it is obvious that the calcium carbonate will sediment with the yeast cells unless removed before centrifugating. We accomplished this by adding dilute HCl, drop by drop, to the tube contents just before centrifugating. The results of this test with varying concentrations of alfalfa extract added, are shown in Series C of Table II and in Chart II. They show that while, as evidenced by the control figures, Medium F is superior to Nægeli solution as a basal medium it is still capable of stimulation by every concentration of the alfalfa extract used and that while the optimum growth obtained is greater than that obtained with Nægeli solution the shape of the curve is essentially the same as with Nægeli solution. These results seem at least to make questionable Fulmer, Nelson, and Sherwood's first contention.

To test their contention regarding the effect of alkali we selected both alfalfa extract and yeast autolysate for experimental materials. Our alkali-treated extracts were obtained in two ways. A portion of the extract was measured out and made up to 10 per cent strength with solid NaOH. This mixture was then autoclaved at 15 pounds above atmospheric pressure for 3 hours. After autoclave treatment the extract was neutralized with HCl to litmus and evaporated to its original concentration. Except for the action of the alkali on the components of the extract the sole change made in composition was an increase in NaCl. 1 cc. portions of the treated extract, diluted to provide varying concentrations, were then combined with 9 cc. of Nægeli solution in one experiment and with Medium F in another. Previous experiments had provided us with the control data (see Table II and Chart II). Table VI and Chart IV give the results.

TABLE VI.

The effect of alkali treatment upon the stimulative powers of alfalfa and yeast extracts.\*

Studies of alfalfa extracts. (C equals the extractable material from 0.4 gm. of dried alfalfa in 1 cc. of water.)

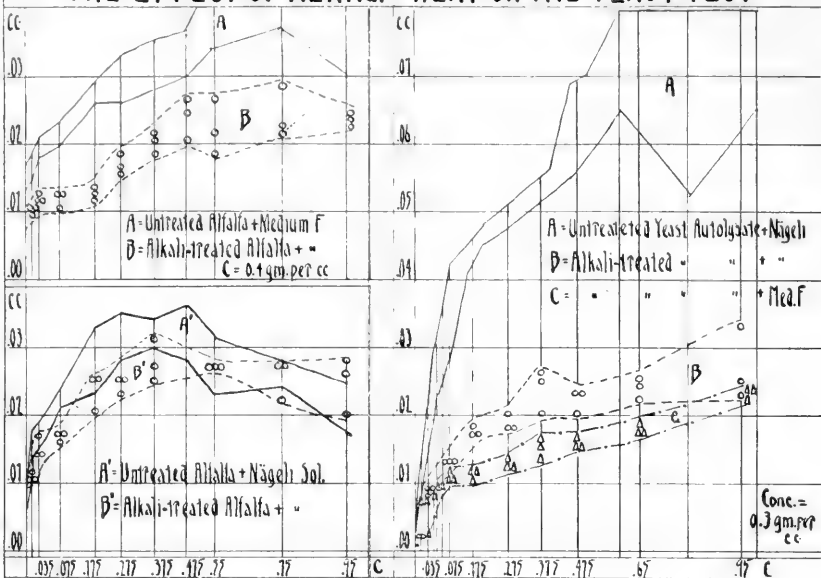
	Concentrations used.	Tests with alkali-d extracts.	Average.	Average untreated extract.
Nägeli solution used.	0.95C	20 26 28	24	20
	0.75C	22 27 27	25	27 See Table III.
	0.55C	27 27 27	27	28
	0.475C	26 27 28	27	32
	0.375C	25 27 31	28	33
	0.275C	23 25 25	24	31
	0.175C	20 25 25	23	28
	0.075C	16 16 17	16	22
	0.035C	14 14 17	15	17
	0.015C	10 10 11	10	16
	Control.	2 2 2	2	2
Medium F used.	0.95	22 23 24	23	43
	0.75	21 22 28	24	45
	0.55	18 22 26	23	39
	0.475	20 24 26	23	32 See Table III.
	0.375	18 20 21	19	31
	0.275	15 16 18	16	29
	0.175	11 12 13	12	27
	0.075	10 12 12	11	22
	0.035	11 11 12	11	19
	0.015	9 10 10	10	19
	Control.	4 4 4	4	15

Studies of yeast autolysate. (C equals 0.75 gm. in 1 cc.)

Concentrations used.	Alkali-treated with Nægeli.	Average.	Alkali-treated with Medium F.	Average.
0.95C	23 25 33	27	22 23 23	22
0.05C	22 24 25	24	17 17 18	17
0.475C	20 23 23	22	15 15 16	15
0.375C	20 25 26	24	13 15 16	15
0.275C	18 18 20	18	12 12 13	12
0.175C	17 17 18	17	11 11 10	11
0.075C	13 13 13	13	10 10 11	10
0.035C	8 9 9	9	8 9 9	9
0.015C	2 2 2	2	2 7 7	5
Control.	2 2	2	3 3 3	3

\* For comparison with untreated yeast autolysate see Chart IV.

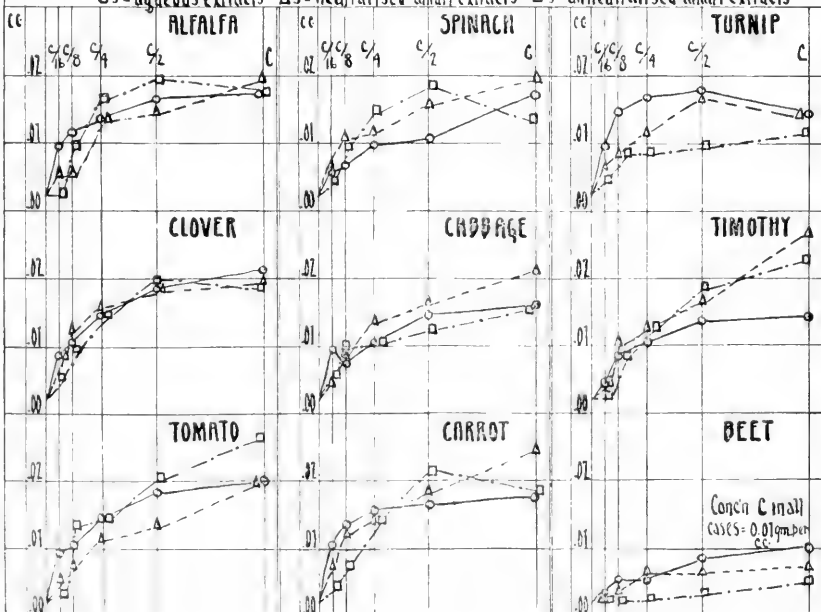
# CHART II. THE EFFECT OF ALKALI + HEAT ON THE YEAST TEST



## THE RESULTS OF AN ATTEMPT TO EVALUATE EXTRACTS OF ALKALI + HEAT

The average of 7 det's. made on each of 7 concentrations is recorded

○ = aqueous extracts    △ = neutralised alkali extracts    □ = unneutralised alkali extracts



When these results are examined carefully the conclusions are not so definite as the figures might at first indicate. They seem to show destruction by the alkali of some of the power but the extent of the destruction is not nearly so great as would be expected if vitamine B formed a large part of the stimulative factors. It is also easy to see why Fulmer, Nelson, and Sherwood reached their conclusion as to practically no destruction if they tested only the higher concentrations, for in the higher concentrations the effect is much less marked, especially in the experiments with Nägeli solution as the basal medium. These facts show up more distinctly in the charted results. We therefore resolved to make another test. If vitamine B is one of the factors and if it is destroyed by alkali the extracts of the Osborne and Mendel series ought to show a marked reduction in stimulative power after alkali treatment. To determine this we made a new set of extracts of those materials. These sets consisted of three series obtained as follows: Series A represents aqueous extracts of 1 gm. of dried material made up with water so that 1 cc. carried the "bios" of 0.01 gm. of dried material. Series B consists of a similar set obtained by extracting 1 gm. of the dried material with 0.1 N NaOH, filtering, neutralizing with HCl to litmus, and diluting until 1 cc. carried the "bios" of 0.01 gm. of dried material. In both these extractions the material was boiled continuously for 3 hours in the extractant. The third series was obtained by taking 50 cc. portions of Series B, adding 5 cc. of 0.1 N NaOH to each portion, heating at 100°C. for 30 minutes, and then testing without neutralizing. We assumed that the order of test effects would be in all cases highest with Series A, Series B coming next, and Series C third. The actual readings are given in Table VII and graphically in Chart IV.

These results fail to confirm the conclusions from Table VI and seem to give excellent confirmation of Fulmer, Nelson, and Sherwood's view that the stimulative factor is not appreciably affected by alkali treatment. These two sets of experiments, therefore, leave that question unsettled.

From these results and from the previous ones it becomes increasingly evident that the growth of the yeast cells, as determined by any one of the methods devised to date, is a resultant of so many different factors, mutually interacting, that to interpret

these growths as a quantitative measure of vitamine content is unjustified. All the evidence except that based on alkali treatment seems to argue in favor of vitamine B as at least one of the

TABLE VII.

Effect of alkali treatment on the Osborne-Mendel preparations. Only the averages are given.

Materials tested.....	Alfalfa.	Clover.	Tomato.	Spinach.	Cabbage.	Turnip.	Carrot.	Timothy.	Beet.	Control.
Full concentration of aqueous extract.....	17	21	20	17	16	14	18	14	10	2
Neutral alkaline extract....	19	19	20	19	21	14	24	26	7	1
Alkaline extract with 5 cc. alkali.....	17	18	26	14	16	11	18	22	5	1
Half concentration of aqueous extract.....	16	18	18	10	14	17	16	13	8	2
Neutral alkaline extract....	14	18	13	15	16	16	18	16	6	2
Alkaline extract with 5 cc. alkali.....	19	19	20	18	12	9	21	18	3	2
Fourth concentration of aqueous extract.....	13	14	14	9	10	16	15	10	5	2
Neutral alkaline extract....	13	15	11	11	13	11	14	13	6	2
Alkaline extract with 5 cc. alkali.....	16	14	15	14	10	8	14	13	2	2
Eighth concentration of aqueous extract.....	11	10	10	6	7	14	13	8	5	2
Neutral alkaline extract....	5	12	7	10	8	8	12	10	4	2
Alkaline extract with 5 cc. alkali.....	9	9	13	9	9	8	7	8	2	2
Sixteenth concentration of aqueous extract.....	9	8	9	5	9	9	11	4	3	2
Neutral alkaline extract....	5	8	5	6	4	6	7	5	3	2
Alkaline extract with 5 cc. alkali.....	3	5	3	5	5	4	4	2	2	2

growth stimulants while the alkali evidence is contradictory and incomplete. There seems, therefore, only one alternative; namely, to attempt to separate and identify the factors concerned in the growth stimulation and if possible devise a medium that



and then allowing the mixture to stand for 24 hours the carbon was filtered off on a suction funnel. This carbon was then washed on the funnel and by shaking repeatedly with distilled water until all adherent material that was removable by this washing was eliminated. The washed carbon was then boiled with glacial acetic acid and the acid extract filtered off through an alundum filter. This acid extract was evaporated to dryness on the steam bath and the residue taken up with distilled water and neutralized. Its volume was made up with water to equal that of the alfalfa extract from which it was derived. This extract in full concentration and in several dilutions was tested with the yeast test and the results are shown in Chart V. When compared with the test strength of the solution from which it was obtained the recovery of the stimulation factor by this method seems to have been rather high. With a portion of this extract we repeated the alkali treatment and obtained an unmistakably destructive effect, though not complete destruction. This result leads us to retain our faith that one of the factors concerned is either vitamine B or one that resembles it somewhat closely in properties (14).

Analyses are now being conducted on the carbon recovered factor to obtain a possible clue as to its nature.

If we picture the action of a factor on a yeast cell we may assume that the process might well consist of three steps; first diffusion, by which it comes in contact with the cell; second, possibly adsorption or at any rate penetration into the cell; and finally, its specific stimulation to protoplasmic production. If this is true the following conditions will exist and influence the growth of the cell; *viz.*, number of factors present, concentration of these factors, and rate of diffusion of factor to cell. It occurred to us that the failure of high concentrations to permit optimum growth might be a matter of diffusion. The following tests were devised to test this view. The two concentrations of alfalfa extract used in the time of incubation test (Table V) were selected, and two sets of tubes prepared as usual. One set was kept perfectly quiet at room temperature, after inoculation with yeast suspension for 5 days. The second set was treated in the same way except that at intervals in the 5 days they were transferred to the shaking machine and shaken a total of 15 hours in all in the 5 days. The



rate of growth in the two sets is shown in Table VIII. Having thus shown that diffusion is an important factor, a second test was devised as follows. Two sets of tubes were prepared as before but the 1 cc. of extract was in one set of tubes placed within a collodion sac suspended in the Nägeli solution. The effect of this process was to reduce slightly the growth rate but there is

TABLE VIII.

The effect of changing diffusion conditions upon the yeast test.

Shaking experiment.										
	Full concentration of alfalfa.					Optimum concentration of alfalfa.				
	Test.				Average.	Test.				Average.
5 days with no shaking.....	10 11 15 11				12	22 25 22 27 22				24
5 days with a total of 15 hrs. shaking.....	22 15 21 16 26 18				20	29 35 39 34				34
Control (still)...	2 2 2				2					
Control shaken..	3 4 4				4					
Collodion bag experiment.										
	Full concentration yeast autolysate extract.					Half concentration yeast autolysate extract.				
	Test.				Average.	Test.				Average.
1 cc. extract mixed with Nægeli solution in tube...	84 80				82	73 71				72
1 cc. extract suspended in collodion sac.....	67 62 60 82				68	64 56 61 61				60
Control.....					2					2

no doubt that the growth factor passes through the membrane; *i.e.*, is highly diffusible through membrane. The results are shown in Table VIII. We are now trying to determine whether by a study of the content of the collodion sacs before and after diffusion, light may be thrown on the nature of the factors. These few experiments are cited merely as illustrating the complexity of factors taking part in a yeast test.

## SUMMARY.

1. When the yeast test is applied to materials already evaluated as to vitamine content by rat-feeding experiments, the results show only approximate agreement. The agreement is more marked, however, when the extracts are dilute.

2. A study of the curve formed by plotting all the determinations obtained with varying concentrations of an aqueous extract of alfalfa shows that the reaction does not give the appearance of a monomolecular reaction. From the control point to the optimum it approximates the shape of the logarithmic curve but to the right of the optimum point there is a distinct decline indicating inhibitory factors in the higher concentrations.

3. A comparison with curves obtained from other extracts shows that the optimum growth varies not only with the concentration but with the nature of the extract tested.

4. A study of the effect of extracts upon growth of yeast cells, using Fulmer, Nelson, and Sherwood's medium F in place of Ngeli solution fails to support their contention that the growth stimulus is purely a matter of concentrations of known constituents.

5. Certain results, following the use of alkali, seem to indicate that if the solution treated is sufficiently dilute, the destructive effect of the alkali will appear; but attempts to verify this with extracts of the materials used in the rat-feeding tests gave most contradictory results. In any case it seems evident that at least some of the factors concerned in the stimulation are not affected by alkali treatment but the data is not sufficiently complete to justify the view that vitamine B is not present and therefore not one of the functioning stimulants.

6. The cumulative effect of the data obtained is to suggest that in its present state the test is distinctly unreliable as a quantitative measure of vitamine content. On the other hand it suggests interesting possibilities as a method for studying the kinds and behavior of growth stimuli.

7. Experiments are cited that illustrate some of the factors that enter into the growth results and a method of analyzing their nature is given.

8. Until a basal medium is worked out that provides an optimum of all the factors except vitamine B the test must be considered of little value in the estimation of true vitamine content.

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## NOTES ON APPARATUS USED IN DETERMINING THE RESPIRATORY EXCHANGE IN MAN.

### I. AN ADAPTATION OF THE FRENCH GAS MASK FOR USE IN RESPIRATORY WORK.

By CAMERON V. BAILEY.

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(Received for publication, May 2, 1921.)

Considerable difficulty has been experienced in finding a suitable breathing appliance to be used with respiration apparatus. To insure absolute rest and normal type of respiration it is essential that the subject suffers no discomfort. The appliance should permit him to breathe through the nose, mouth, or both as is his custom; otherwise abnormal conditions are imposed, and his attention being focused on the respiratory act, unnatural breathing results.

Appliances necessitating the insertion of tubes into the mouth have the additional defect of causing salivation, with a resulting frequency of deglutition and, in the closed circuit apparatus, the possible swallowing of oxygen. Of the appliances at present in use, the half mask used by Boothby and Sandiford<sup>1</sup> presents none of these defects. In practice, however, difficulties arise in the use of the half masks; the engaging surfaces are small and the bony support of the tissues deficient; in the absence of molar teeth, the cheeks sag inwards during inspiration and leaks are apt to occur. In the attempt to avoid leaks the mask is frequently applied so firmly to the face that extreme discomfort results.

The rubber gas mask used in the French army is admirably suited to this work.<sup>2</sup> It is made of thick rubber, covers the whole

<sup>1</sup> Boothby, W. M., and Sandiford, I., *Laboratory manual of the technic of basal metabolic rate determinations*. Philadelphia and London, 1920, 35.

<sup>2</sup> The gas masks and valve attachments may be obtained from the C. M. Sorensen Co., 177 East 87th Street, New York.

face, and presents broad surfaces which closely engage the forehead, sides of the face, and jaw. The tissues in these regions are well supported by the bony framework of the face and the mask readily adapts itself to these fixed surfaces. It is held in place by elastic straps passing around the head. In emaciated subjects, leaks may occur above or below the zygoma, in this area the pull of the straps is in the same plane as the surface of the face. In such rare instances the leaks are readily overcome by placing 6 inch rubber sponges over these areas of the mask and binding them firmly in place with a 3 inch bandage. In this mask, the incoming air is directed

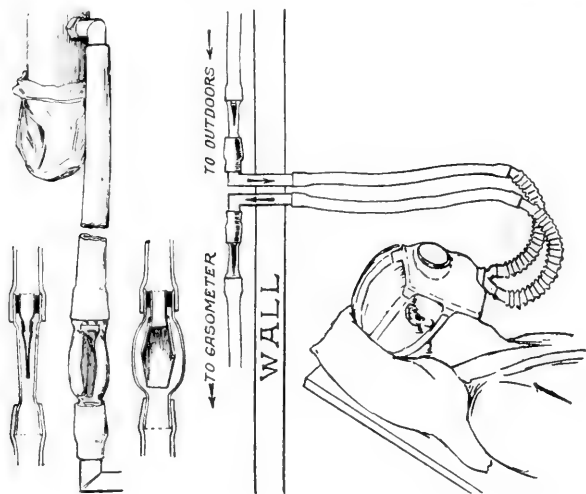


FIG. 1.

upwards towards the windows, the opening of the expiratory tube being opposite the nose and mouth. In this way the space is perfectly ventilated and no discomfort results. Rubber flutter valves are eminently satisfactory for use with these masks. They are conveniently enclosed in flattened glass tubes introduced as near the mask as practicable. A satisfactory arrangement of mask and valves for the Tissot method is shown in Fig. 1. The subject reclines in a wheel chair in the rest room, the tubes pass through the wall into the laboratory where the valves are mounted, the tubes then lead directly to the air supply and to the gasometer. A window permits the operator in the laboratory to observe the

subject, while at the same time he can follow the respirations by watching the movement of the valves and start and stop the test by turning the 3-way valve on the gasometer.

The incoming air enters the room through a 4 inch pipe, the end of which is closed by a rubber bathing cap held in place by a rubber band. This takes up the pressure of gusts of wind and prevents it blowing through the valves. A 2 foot length of 24 mm. rubber tubing leads from the large pipe to the inspiratory valve, this is for the purpose of trapping any expired air which might backlash through the valve. In using the mask with closed circuit apparatus double tubes lead to the oxygen reservoir, the expiratory tube, containing a flutter valve, leads through the  $\text{CO}_2$  absorbed. With portable apparatus the valve case can be made of transparent celluloid which is not readily broken. In this type of apparatus the use of the mask with a single tube and without valves is unsatisfactory as the dead space is increased to too great an extent.





## NOTES ON APPARATUS USED IN DETERMINING THE RESPIRATORY EXCHANGE IN MAN.

### II. A SAMPLING BOTTLE FOR GAS ANALYSIS.

By CAMERON V. BAILEY.

(From the Laboratory of Pathological Chemistry (Respiratory Division),  
New York Post-Graduate Medical School and Hospital, New York.)

(Received for publication, May 2, 1921.)

This appliance is designed for readily collecting and holding samples of gas and for transferring them to a gas-analyzing burette without danger of dilution or loss of mercury.

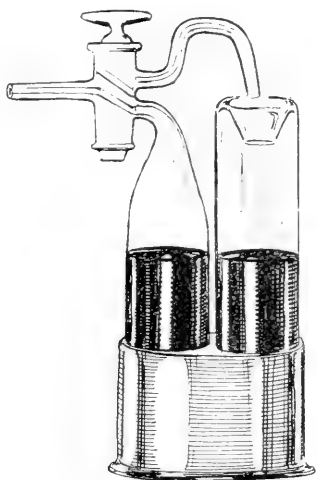


FIG. 1.

The bottle<sup>1</sup> shown in Fig. 1 is 19 cm. high and has a gas capacity of 60 cc.; this is a convenient size for use in conjunction with a gasometer and the Henderson<sup>2</sup> modification of the Haldane gas analyzer.

<sup>1</sup> This bottle may be obtained from the C. M. Sorensen Co., 177 East 87th Street, New York.

<sup>2</sup> Henderson, Y., *J. Biol. Chem.*, 1918, xxxiii, 31.

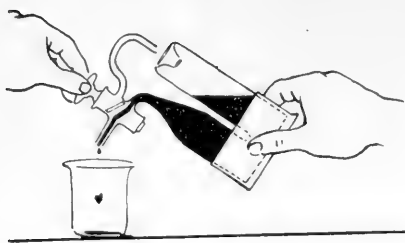


FIG. 2.

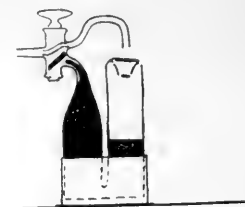


FIG. 3.

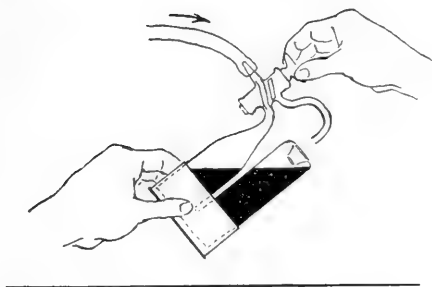


FIG. 4.

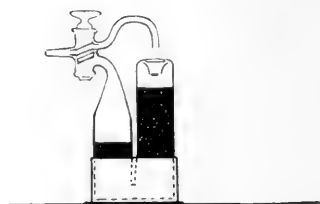


FIG. 5.

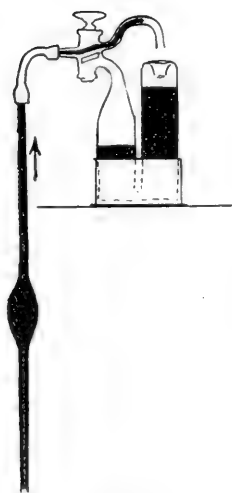


FIG. 6.

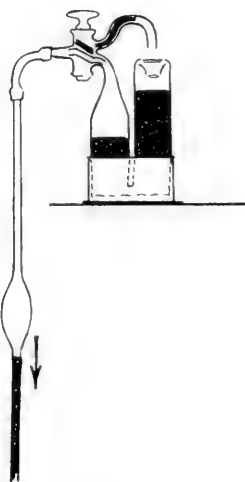


FIG. 7.

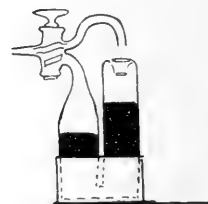


FIG. 8.

The bottle as illustrated consists of parallel glass cylinders communicating at the bottom through a small opening. The cylinders are firmly cemented into a metal case which forms a base. The top of one cylinder has a funnel-shaped inversion; the other tapers off to a 2 mm. capillary tube to which a 2-way stop-cock is fused. The second opening in the stop-cock communicates with a capillary tube which bends over the inverted top of the first cylinder. The stop-cock terminates in a straight spout of capillary tubing 3 cm. long. The appliance is half filled with mercury, and before use is flushed out with 1 per cent sulfuric acid.

The series of small drawings illustrate the manipulation of the bottle.

Fig. 2. It will be seen that by tilting the bottle forward, the mercury displaces the air in the gas compartment and in the barrel of the stop-cock.

Fig. 3. If the stop-cock be completely reversed while the bottle is in this position, these parts remain filled with mercury and the spout is in communication with the bent capillary tube through which the gas to be sampled is blown.

Fig. 4. The stop-cock is returned to its original position and the sample of gas is drawn into the bottle by tilting it backward.

Fig. 5. By turning the stop-cock while the bottle is in the last position the sample is trapped in the compartment under pressure and the spout again communicates with the curved capillary tube.

Fig. 6. The sampling bottle is now placed on a shelf over the analyzer framework; the spout connected to the top of the gas burette and the mercury in the latter forced through this communication rendering it free from air.

Fig. 7. The stop-cock is now turned to the first position, the mercury in the burette lowered, and a sample of the gas is drawn into the gas burette.

Fig. 8. The stop-cock is again reversed and sufficient gas remains for five additional analyses.



# STUDIES ON THE DIGESTIBILITY OF PROTEINS IN VITRO.

## II. THE RELATIVE DIGESTIBILITY OF VARIOUS PREPARATIONS OF THE PROTEINS FROM THE CHINESE AND GEORGIA VELVET BEANS.

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(Received for publication, May 19, 1921.)

The ill effects (1, 2) which have sometimes followed the use of velvet bean meal as the principal source of protein in stock feeds have lead to an investigation by Miller (1) in which he isolated a toxic substance, 3, 4-dihydroxyphenylalanine, from the seed of the Georgia velvet bean, *Stizolobium deeringianum*. This discovery seemed at the time of its publication to furnish a sufficient explanation of the objectionable properties exhibited by these beans. But feeding experiments recently made in this laboratory (3), while they tend to confirm the supposition that the velvet beans contain a toxic substance, have brought out also the interesting fact that the isolated proteins prepared from the Chinese (*Stizolobium niveum*) and Georgia velvet beans by dialyzing their saline extracts are no better tolerated than is the bean meal. There must, then, be some limiting factor other than the dihydroxyphenylalanine *alone*. Amino-acid deficiency cannot account for the failure to promote growth, for analyses (4, 5) have shown these proteins to be adequate except possibly with respect to cystine; and no improvement resulted from the addition of this amino-acid. Further, the proteins as obtained by coagulation gave normal growth. The most reasonable hypothesis to account for these observations seemed to be that the proteins in the raw state as prepared by dialysis are not sufficiently digestible to be available for animal nutrition, while the coagula were cooked, by the boiling incident to the process of preparation, enough to render them sufficiently digestible. The primary purpose of the experiments described in this paper was to test the correctness of this

conclusion. We wished also to secure further data on the extent to which our results derived from experiments *in vitro* run parallel with those of growth and utilization tests made with animals.

The method employed was essentially that of Waterman and Johns (6), described in the first paper of this series. Inasmuch as proteins from different sources were to be compared in the present case, the results were calculated on the basis of the total amino nitrogen of the protein, minus the free amino nitrogen ( $\frac{1}{2}$  the lysine nitrogen), as suggested in the first paper. The formula for the calculation of the percentage of digested nitrogen becomes, then,

$$D N = \frac{N_d - N_b}{N_a} \times 100 \text{ where}$$

$N_d$  = mg. amino N found in the reaction mixture after digestion, corrected for the blank due to the reagents used in the Van Slyke amino N apparatus.

$N_b$  = mg. amino N produced by self-digestion from the enzymes in a blank digestion; corrected as above.

$N_a$  = mg. total amino N, derivable by complete hydrolysis from the protein, minus one-half the lysine nitrogen.

The experiments confirm our theory based upon the results of the feeding experiments, that the failure of raw dialyzed Chinese velvet bean protein was due to partial indigestibility. The figures obtained for the digestibility of the coagulated proteins of the Chinese and Georgia velvet beans are nearly twice as great as those given by the uncooked dialyzed proteins in either case, and agree well with those yielded by cooked phaseolin (6) and by casein (Table IV). The figures found for the raw dialyzed proteins are about 8 per cent lower than those given by *raw* phaseolin. This corresponds well with the growth experiments in these two cases. It seemed almost certain that the difference between the raw dialyzed and the coagulated velvet bean proteins was due simply to the cooking involved in the preparation of the latter material. There remained, however, the possibility that the coagula contained protein not precipitable by dialysis and that their greater percentage of digestible nitrogen might have been due in part to these other proteins. In order to eliminate this question we made cooked preparations of the protein obtained by dialysis from each of the two species of velvet beans and have compared their digestibility with that of the corresponding raw dialyzed pro-

teins. Both cooked preparations showed a digestibility agreeing closely with those of the corresponding coagula.

In addition to the difference in the percentages of digestion nitrogen, certain purely qualitative indications were observed which also point to a marked superiority in digestibility of the coagulated and the cooked dialyzed preparations over those of the raw dialyzed proteins. In the first place, there appeared in the digests of the raw dialyzed samples, on neutralizing the acid after digestion with pepsin, a bulky, light gray, flocculent precipitate apparently consisting of incompletely peptonized protein. The coagulated and the cooked dialyzed protein formed only a small precipitate at this point. Also, when the digests are heated, to inactivate the enzymes at the end of the digestion period with trypsin, a small amount of coagulation always occurs, although the solution is distinctly alkaline. In the present case this precipitate was always considerably greater in the reaction product of the raw dialyzed, than it was in those of the cooked or the coagulated proteins. Again, in making the determinations of amino nitrogen on the filtrate from these reaction mixtures, we found that the digestion products of the raw dialyzed samples foamed persistently in the Van Slyke apparatus, filling both the deaminizing bulb and the gas burette with a thick froth which made it almost impossible to carry out the analysis accurately, unless diphenyl ether or some other foam inhibitor was used. This behavior of the digests of the uncooked preparations is very characteristic of solutions containing unhydrolyzed protein. The digests of the cooked and the coagulated samples, on the other hand, gave practically no trouble of this sort. Finally, if the filtered, slightly alkaline digestion products were made just acid with acetic acid a further precipitate was produced, and, here again, its amount was considerable in the case of the raw dialyzed preparations and very slight, a mere cloud hardly greater than that yielded by the blank digestion, in the case of the cooked or the coagulated protein.

This combined evidence leaves little doubt that the difference in digestibility between the raw dialyzed and the coagulated velvet bean proteins is due simply to cooking. Also, the experiments as a whole tend decidedly to confirm our supposition that incomplete digestibility is one of the limiting factors in the nutritional

failure of raw velvet bean meal and the only limiting factor in the similar failure of the protein isolated by dialysis.

#### EXPERIMENTAL.

*Preparation and Analysis<sup>1</sup> of the Proteins.*—The coagulated and the dialyzed proteins from the Chinese velvet bean were made<sup>2</sup> in accordance with the direction of Johns and Finks (4). The dried preparations were ground to pass a 100 mesh sieve, the powdered material was exposed to a filtered current of air to come to equilibrium with atmospheric moisture, and the total nitrogen, not corrected for ash or moisture, was determined. The total amino nitrogen of each preparation, minus its *free* amino nitrogen ( $\frac{1}{2}$  the lysine nitrogen), was then calculated from the figures of the Van Slyke analyses given in the paper referred to above.

The coagulated Georgia velvet bean protein was prepared like that from the Chinese variety. The dialyzed protein was precipitated directly from the saline extract without preliminary fractionation by ammonium sulfate. Both of these preparations, therefore, contained a mixture of the  $\alpha$ - and  $\beta$ -globulins of the Georgia bean, and in addition to these the coagulum contained the traces of albumin present in the extract. The dried mixtures were prepared for analysis and their total nitrogen determined as above described; but the presence in each of more than one protein made necessary a different procedure in the estimation of the amino nitrogen. An average of the amino nitrogen figures, taken from the nitrogen distribution given by Johns and Waterman (5) for the  $\alpha$ - and  $\beta$ -globulins, corrected for free amino nitrogen as above, and weighted according to the relative yields of the two proteins, was used as the basis of the calculation in the case of the dialysis product. The maximum error which could be introduced into the calculation of the digestion nitrogen by the use of the value thus derived would not be significant for the purposes of the present experiments. The coagulum, however, contained albumin, the

<sup>1</sup> The elementary analysis of the preparations was made by S. Phillips of this laboratory.

<sup>2</sup> This material was prepared by C. E. F. Gersdorff of this laboratory. Both varieties of the beans were furnished by the Bureau of Plant Industry, United States Department of Agriculture.



relative quantity of which could not be estimated with any approach to accuracy. A sample of about 3 gm. of this material was hydrolyzed, therefore, and freed from ammonia as in the determination of the distribution of nitrogen by Van Slyke's method. After expelling the ammonia, the hydrolysate was acidified with acetic acid, concentrated to a syrup, transferred to a 200 cc. graduated flask, and made up to the mark with distilled water. The amino nitrogen in this solution was then determined by means of the Van Slyke apparatus, and the  $N_a$  calculated from the value thus obtained.

*Digestion with Pepsin.*—The procedure was the same as that previously described (6), except that 0.1 N hydrochloric acid was substituted for the 0.1 N sulfuric acid used in the first experiments. Two or three samples, approximately 0.500 gm., of each of the proteins to be compared were suspended each in 25 cc. of 0.1 N hydrochloric acid, 25 cc. of a 0.2 per cent solution of pepsin in the same reagent were added, and the mixtures incubated for  $1\frac{1}{2}$  hours at  $37^\circ$ . The raw dialyzed proteins from both the velvet beans readily dissolved in the dilute acid, while the coagulated and the cooked dialyzed proteins were not completely dissolved until near the end of the digestion period with pepsin.

*Digestion with Trypsin.*—After the peptic digestion the 50 cc. of 0.1 N acid were neutralized with 5 cc. of N sodium hydroxide and 5 cc. of a 6 per cent solution of trypsin in 0.1 N sodium hydroxide were added. The reaction mixtures were then returned to the incubator and digested for  $2\frac{1}{2}$  hours at  $37^\circ$ . The activity of the enzymes was then destroyed by heating to  $80^\circ$  on the steam bath, the solutions were cooled and filtered, and amino nitrogen was determined. With each set of experiments a blank digestion of the enzymes alone without any added protein was carried out in exactly the same way as were the digestions of the samples, in order that the amino nitrogen set free by the self-digestion of the enzymes might be determined and a correction made for it. The resulting data, together with the percentages of digested nitrogen calculated according to the formula given above, will be found in Tables I, II, and III. The average digested nitrogen values for casein, cooked and raw phaseolin, and for each of the six velvet bean preparations studied are presented for comparison in Table IV.

**TABLE I.**  
*Comparative Digestibility of Coagulated and Dialyzed Chinese Velvet Bean Proteins.*

Preparation.	Amount of sample (protein).	Combined amino N in sample ( $N_a$ ).	Amino N after digestion ( $N_d$ ).	Amino N of blank digestion ( $N_b$ ).	Digestion N, calculated on basis of $N_a$ .
	mg.	mg.	mg.	mg.	per cent
Coagulated.....1	501.3	51.13	48.09	18.24	58.0
2	501.8	51.18	48.59	18.24	59.1
Average.....					58.6
Dialyzed.....1	502.1	54.23	30.51	18.24	22.4*
2	502.0	54.22	33.99	18.24	28.9
Average.....					28.9
Coagulated.....3	500.9	51.09	47.72	19.20	55.8
4	501.2	51.12	47.72	19.20	55.8
Average.....					55.8
Dialyzed.....3	501.3	54.15	35.40	19.20	29.9
4	501.2	54.14	35.90	19.20	30.8
5	501.2	54.14	35.73	19.20	30.5
Average.....					30.4
General average.					per cent
Dialyzed.....					29.7
Coagulated.....					57.4

\* Not included in the averages.

**TABLE II.**  
*Comparative Digestibility of Coagulated and Dialyzed Georgia Velvet Bean Proteins.*

Preparation.	Amount of sample (protein).	Combined amino N in sample ( $N_a$ ).	Amino N after digestion ( $N_d$ ).	Amino N of blank digestion ( $N_b$ ).	Digestion N, calculated on basis of $N_a$ .
	mg.	mg.	mg.	mg.	per cent
Coagulated.....1	501.0	53.96	48.56	18.80	55.2
2	501.0	53.96	48.89	18.80	55.8
Average.....					55.5
Dialyzed.....1	501.5	56.02	36.50	18.80	31.6
2	501.6	56.03	36.66	18.80	31.9
Average.....					31.8

TABLE III.

*The Effect of Cooking upon the Digestibility of Dialyzed Velvet Bean Proteins.*

Preparation.	Amount of sample (protein).	Combined amino N in sample ( $N_d$ ).	Amino N after digestion ( $N_d$ ).	Amino N of blank digestion ( $N_b$ ).	Digestion N, calculated on basis of $N_a$ .
	mg.	mg.	mg.	mg.	per cent
Georgia.					
Dialyzed,					
cooked*.....1	501.1	54.38	48.53	18.86	54.5
2	501.3	54.40	48.65	18.86	54.8
Average.....					54.7
Georgia.					
Dialyzed, raw..3	502.3	56.10	37.11	18.86	32.5
4	502.0	56.07	37.34	18.86	32.9
Average.....					32.7
Chinese.					
Dialyzed,					
cooked*.....1	501.2	52.18	48.02	17.65	58.2
2	500.6	52.12	47.62	17.65	57.5
Average.....					57.9

\* An aqueous suspension was boiled 2 hours, evaporated to apparent dryness on a steam bath, and ground to 100 mesh powder.

## DISCUSSION.

From the properties of the velvet bean proteins brought out by these experiments we may conclude, in the absence of experimental evidence to the contrary, that our hypothesis regarding partial indigestibility as the limiting factor in the failure of the raw dialyzed Chinese velvet bean protein to promote growth is the correct one. The nutritional inadequacy of the uncooked protein, together with the fact that a normal growth rate can be secured with the coagulated protein, is then to be referred to difference in digestibility alone. The raw ground seed would have the double disadvantage of toxicity and non-available protein content, while the cooked meal would probably still contain dihydroxyphenyl-alanine. Our experiments also justify us in predicting with some confidence that the raw dialyzed protein from the Georgia velvet

bean will behave in nutrition experiments like that from the Chinese variety; and that cooking under conditions similar to those described above will render either of these dialyzed preparations capable of supplying the protein requirement of normal growth.

TABLE IV.

*Comparative Summary of Digestibility Experiments in Vitro and Nutritional Experiments in Vivo, on the Velvet Bean and Other Proteins.*

Protein.	Digestibility. <i>in vitro</i> (average).	Result of growth experiments.
	<i>per cent</i>	
Chinese velvet bean coagulum.....	57.4	Normal growth (2).
Georgia " " " .....	55.5	" " (2).
Chinese " " , dialyzed protein.	30.4	Supports neither growth nor life (2).
Georgia " " " " .....	32.3	Not tried.
" " " " cooked dialyzed protein.....	54.7	" "
Chinese velvet bean, cooked dialyzed protein.....	57.9	" "
Phaseolin, raw*.....	39.5	Does not support growth, with or without cystine (7).
Phaseolin, cooked†.....	58.7	Normal growth when supplemented with cystine (7).
Casein.....	61.4	Normal growth.

\* Recalculated from Table II, first paper (6).

† New determination, using hydrochloric in place of sulfuric acid in the pepsin digestion.

With regard to the relation between the values obtainable in determinations of the type under consideration, and the results of growth experiments with animals, the figures presented in Table IV give some indication. Six of the nine preparations listed have been fed as the sole source of protein in an otherwise adequate diet. Of this group, four produced normal growth in animals and gave relatively high digestibility values *in vitro*. Cooked phaseolin required the addition of cystine, it is true; but cystine did not improve the raw phaseolin diet, and even without

the addition of cystine the cooked phaseolin maintained the weight of the animals for some time. The other two proteins tested supported neither growth nor life, and they showed a relatively low digestibility *in vitro*. Finally, the greatest digestibility so far observed in our experiments is that of casein, the principal component of the natural food of young mammals. Thus all the evidence at present available supports the assumption tentatively put forward in our first paper on this subject, that an indigestibility sufficient to interfere seriously with the capacity of a protein to furnish the amino-acid requirement of normal growth may readily be demonstrated by this method.

Concerning the relation existing between these estimations of digestibility *in vitro* and absolute digestibility as determined in the animal organism, however, we have as yet no reliable information beyond the fairly safe inference that figures for different proteins as found by the two methods may be expected to vary in the same direction and with a rough proportionality. Any direct arithmetical ratio is probably precluded by the subtraction of the considerable blank correction for the self-digestion of the enzymes in calculating the results of the estimations *in vitro*, since, of course, this self-digestion is considerably greater in the blank digestion where no substrate is present than it is in digests containing a protein sample. It is even possible that self-digestion is so far retarded by the substrate that a less error would be introduced by omitting this correction entirely than by including it in the calculation. Bayliss (8) reports an experiment in which trypsin, acting upon casein as a substrate, showed "no appreciable destruction up to eight hours." It is of interest to note in this connection that if the data of our experiments be calculated *without subtracting the blank* they yield figures much like those obtained by the absolute method in similar cases. Various experiments with casein, for instance, when calculated on this basis gave values ranging from 99.5 to 100.2 per cent. The values for the cooked and the coagulated velvet bean proteins lie between 90 and 100 per cent on this basis, and those for the raw velvet bean proteins would be about 65 to 70 per cent. The figure 73.6 has been given as the digestion coefficient for the nitrogen of velvet bean meal (beans and pods) when fed to steers (2). Until we have further experimental data bearing upon this point,

however, we prefer to regard our results as purely comparative, and to include the blank in the calculation.

The chemical nature of the indigestibility of certain proteins, which manifests itself in animals by a failure to promote growth and *in vitro* by a relatively low yield of amino nitrogen set free by proteoclastic enzymes, also presents a problem of which little is known. The most probable answer to the question seems to be suggested by the work of Fischer and Abderhalden on the hydrolysis of polypeptides by proteoclastic enzymes. These investigators demonstrated that certain polypeptides are hydrolyzable by trypsin while others, containing the same amino-acid residues united in a different order, are not attacked at all. The presence of one or more of the essential amino-acids in the form of such stable combinations would, of course, explain the failure of a protein to promote growth, and would lower the yield of amino nitrogen set free from the protein by enzymes *in vitro*. A study of the undigested residue precipitable by dilute acid from the solutions remaining after our digestion experiments may throw some light upon this point; and it is intended to make such a study the basis of a future report.

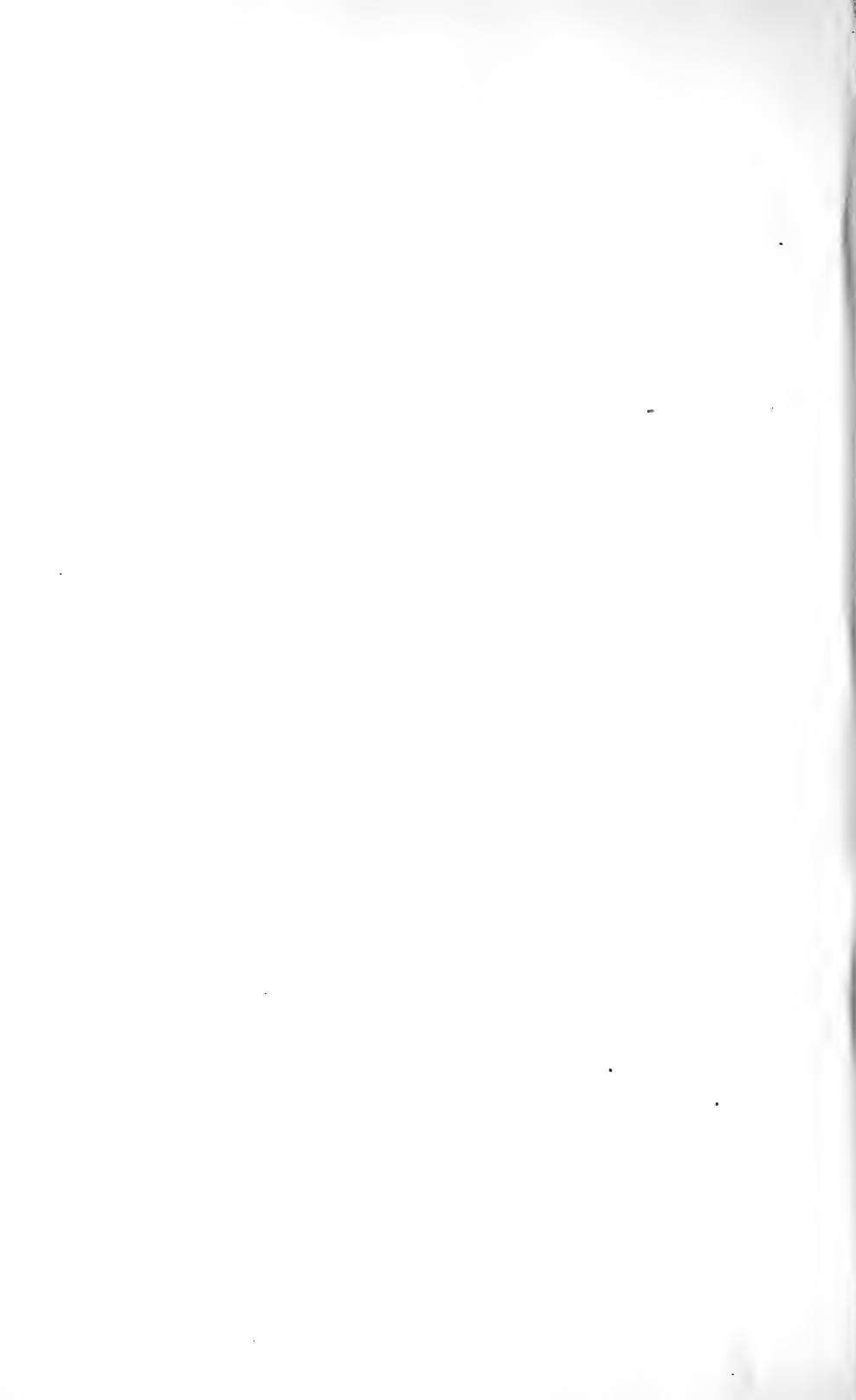
#### SUMMARY.

Estimations of the comparative digestibility *in vitro* by the method of Waterman and Johns of six preparations of the proteins of the Chinese and Georgia velvet beans indicate: (1) That partial indigestibility is the limiting factor in the failure of raw dialyzed Chinese velvet bean protein to promote growth, and that the normal growth secured with the protein prepared by coagulation from either bean is probably to be attributed to an increase in digestibility brought about by the boiling incident to the preparation of the latter material; and (2) that cooking under the conditions described renders the dialyzed protein from either seed as digestible as the coagulated product, and that probably, therefore, these cooked dialyzed proteins will support growth as well as do the coagulated proteins. The double disadvantage of toxicity and non-assimilable protein content amply explains the behavior of the raw ground bean, while the cooked meal probably still contained dihydroxyphenylalanine.

The experiments also support the contention that the results of such estimations run parallel with the results of growth experiments, in as far as differences in the digestibility of the proteins are concerned.

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## THE METABOLISM OF NITROBENZALDEHYDES AND NITROPHENYLACETALDEHYDE.

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(Received for publication, March 26, 1921.)

Several investigators have shown that the metabolism of the *o*-, *m*-, and *p*-nitrobenzaldehydes is entirely different in the body of the dog from that found in the organism of the rabbit, and moreover, that the fate of the *o*-nitrobenzaldehyde is entirely different from that of the *m* and *p* compounds in the same animal organism.

We thought it of interest to determine the fate of these three compounds in the human body, and also to include in the work *p*-nitrophenylacetaldehyde, which, up to the present time, had not been employed in such experiments.

### *o*-Nitrobenzaldehyde.

The pure substance,<sup>1</sup> (melting point, 44°C.), in capsules, was ingested by a man in 2 gm. doses. Because of the toxicity of the compound, only two such doses could be administered. The urine was collected, evaporated to a small volume, acidified with sulfuric acid until the urine showed an acid reaction to Congo red, and repeatedly extracted with ether; the ether was evaporated to dryness and the oily residue taken up with water. After standing 2 days, crystals were recovered from the water solution and dried in a desiccator over sulfuric acid. The melting point of the crystals, 142–144°, showed the substance to be *o*-nitrobenzoic acid. After the first 2 gm. dose of the aldehyde, 1.7 gm. of the acid were isolated from the urine, while after the second dose, 1.8 gm. were found, thus giving a yield of, respectively, 77 and 81 per cent of the substance fed.

<sup>1</sup> The *o*-nitrobenzaldehyde used in this work was furnished by Dr. C. G. Derrick of the National Aniline Works.

Two doses of 2 gm. each of the *o*-nitrobenzaldehyde were fed to a dog of 10.5 kilos body weight. From the urine of the dog we isolated 75 and 80 per cent of the substance in the form of *o*-nitrobenzoic acid.

It appears, therefore, that the fate of the *o*-nitrobenzaldehyde in the human body is much the same as in the organism of the dog.

These results agree with those of Sieber and Smirnow (1), and Cohn (2), who used dogs for their experiments.

Cohn, however, when using rabbits for the same work, was unable to recover more than 10 per cent of the substance fed in the form of *o*-nitrobenzoic acid. We repeated this work on rabbits and were able to confirm his results.

*o*-Nitrobenzaldehyde was found to be much more toxic than the corresponding *m* and *p* compounds. A man of 70.5 kilos body weight, after receiving a 2 gm. dose showed every sign of intoxication, followed by marked albuminuria.

#### *m*-Nitrobenzaldehyde.

The same subject ingested three doses of 2 gm. each of this compound within a period of 96 hours. The urine was collected and treated as in the previous experiment. 4.21 gm. of a substance melting at 139–141° and 2.55 gm. of a substance melting at 160–162° were recovered from the urinary extract. The former substance, *m*-nitrobenzoic acid, as shown by its melting point, represented 63.51 per cent of the *m*-nitrobenzaldehyde fed, while the latter substance, *m*-nitrohippuric acid, is equal to 24.83 per cent of the substance fed, a total of 88.34 per cent.

After 6 gm. of the aldehyde were ingested by a second individual, the ratio of *m*-nitrobenzoic acid to *m*-nitrohippuric acid excreted was entirely different. In this case, about 75 per cent of the aldehyde was found in the urine as *m*-nitrobenzoic acid and only 5 per cent as *m*-nitrohippuric acid. The *m*-nitrobenzaldehyde proved entirely non-toxic, apparently causing no inconvenience to the subjects in doses as large as 5 and 6 gm.

Previous work (1) on dogs has shown that after feeding this substance, *m*-nitrobenzoic acid and *m*-nitrohippuric acid could be recovered from the urine, while after feeding to rabbits (2), an entirely different substance, *m*-acetylaminobenzoic acid was found in the urine.

*p*-Nitrobenzaldehyde.

This substance, in capsules, was ingested by a man in 2 gm. doses. A mixture of the corresponding benzoic and hippuric acids was obtained. Out of a total of 4 gm. taken by the subject, 62 per cent was isolated from the urine as *p*-nitrobenzoic acid and 19 per cent as *p*-nitrohippuric acid.

*p*-Nitrophenylacetaldehyde.

This substance, prepared according to the method of Lipp (3), by the treatment of *p*-nitrophenyl- $\beta$ -chlorolactic acid with sodium carbonate, melted at 84–86°.

The *p*-nitrophenylacetaldehyde was fed to rabbits in 1gm. doses with apparently no ill effects. The urine was collected for 36 hours and treated as usual, except that extraction was first carried out with ether, followed later by two extractions of the evaporated urine with alcohol and then with ethyl acetate. From the ether extract we obtained a yellow oil on evaporating almost to dryness. This oil was difficultly soluble in hot water and after standing in the ice box for 48 hours crystallized out as yellow needles melting when dry at 151–153°. This substance was proven to be *p*-nitrophenylacetic acid by its melting point as well as by its titration value.

After feeding 4 gm. of *p*-nitrophenylacetaldehyde to rabbits, 3.86 gm. of *p*-nitrophenylacetic acid, corresponding to 88 per cent of the substance fed, were recovered from the urine. None of the substance fed, or any of its derivatives, could be found in either the ethyl acetate or alcohol extracts.

5 gm. of this substance were again fed to rabbits, 4.23 gm. of *p*-nitrophenylacetic acid, corresponding to 77.1 per cent of the substance were recovered from the urine.

5 gm. of the substance in capsules were fed to a dog and the urine treated in the same manner as the rabbit urine. From the ether extract 3.92 gm. of *p*-nitrophenylacetic acid were recovered, or 71.35 per cent of the aldehyde fed. No *p*-nitrophenacetic acid nor any derivative of *p*-nitrophenylacetic acid could be found in either the alcohol or ethyl acetate extracts. These extracts were evaporated to dryness, the residue was redissolved in water and fractions of this solution were strongly acidified with sulfuric acid

and boiled for 2 hours. These were then cooled and extracted with ether to determine the presence of any *p*-nitrophenylacetic acid which might previously have escaped extraction in the form of an insoluble conjugation product. The absence of the acid at this point shows that no conjugation product of the acid existed in the urine and that only an oxidation of the aldehyde to an acid had resulted from the passage through the body of the dog.

A man ingested a total of 5 gm. of the *p*-nitrophenylacetaldehyde with much the same results as were previously shown for the dog and rabbit. In this case 70.13 per cent of the aldehyde was excreted in the form of *p*-nitrophenylacetic acid.

Thus, in each case where the *p*-nitrophenylacetaldehyde was fed, there was only an oxidation of the aldehyde to the carboxyl group, which is entirely comparable to the action of the three nitrobenzaldehydes; but in no case was there a reduction of the nitro group to an amino group as was found to be the case when *m*- and *p*-nitrobenzaldehydes were fed to the rabbit. There was also no conjugation of the carboxyl group with glycocoll as was seen when *m*- and *p*-nitrobenzaldehydes were fed to dogs and which was to be expected, as we (4) have previously shown that *p*-nitrophenylacetic acid when fed to dogs is to some extent combined with glycocoll and excreted as *p*-nitrophenaceturic acid.

#### CONCLUSION.

The fate of *o*-, *m*-, and *p*-nitrobenzaldehydes in the human body was much the same as that previously shown for the dog. In each case oxidation took place with the formation of the corresponding acid. In the case of the *o* compound about 90 per cent was excreted as the *o*-nitrobenzoic acid, while the *m* and *p* compounds were excreted to a large extent as the *m*- and *p*-nitrobenzoic acids, but also to a small extent combined with glycocoll and excreted as *m*- and *p*-nitrohippuric acids. In no case was there a reduction of the nitro group.

*p*-Nitrophenylacetaldehyde was fed to rabbits, dogs, and a man. In each case there was only an oxidation to the corresponding acid, with no reduction of the nitro group or combination with either glycocoll or glutamine.

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## FAT-SOLUBLE VITAMINE.

### VIII. THE FAT-SOLUBLE VITAMINE CONTENT OF PEAS IN RELATION TO THEIR PIGMENTATION.\*

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(Received for publication, May 17, 1921.)

In the classification of naturally occurring foods which enter into the make-up of the human dietary the seeds were early given a position of rather uniform value. Thus in 1919 McCollum (1) makes the statement:

"By the application of the biological method of analysis of a food-stuff to each of the more important seeds employed in the nutrition of man and animals, the fact was brought to light that they all resemble each other very closely in their dietary properties. The list of seeds examined included,—wheat, corn, rice, rolled oats, rye, barley, kaffir corn, millet seed, flaxseed, pea and both the navy and the soy bean. . . . All are, with the exception of millet seed, below the optimum in their content of the dietary factor, fat-soluble A."

From our present knowledge it is very evident that this statement merits considerable qualification, for while seeds as a class are apparently never as rich in the fat-soluble vitamine as the leafy parts of plants yet some contain considerable amounts of this vitamine, and among these millet seed, according to our investigations, does not occupy a place of special prominence. In fact, though such varieties of millet may be in existence we have never had as uniform success with these as a source of fat-soluble vitamine as we have had with yellow Indian corn.

That there are other instances where exception must be taken to this statement was brought out in the further development of

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our theory that the fat-soluble vitamine is biologically related to certain yellow plant pigments.

We have already shown that in Indian corn (2) those varieties carrying yellow pigment are rich in the vitamine while those free from it contain very little of this constituent. Further investigation has brought out the fact that in butter fat and in beef fat (3), while there is no absolute parallelism between vitamine and pigment content, yet probably due to their simultaneous occurrence in nature as well as resemblance in properties, where the pigmentation is high the tendency for high vitamine content obtains.

In this paper it is desired to bring out the relation of vitamine and pigment content that has been found to hold among different varieties of edible peas.

#### EXPERIMENTAL.

Six samples of peas were used for the comparisons. Two were purchased in the open market, the one as a sample of split peas sold for cooking purposes having been practically freed from all pericarp; the other was a small green pea of an unknown variety which is commonly used in pigeon feeding. The remaining samples were purchased from a local seed house as pure bred varieties.

Pigment estimations were made by extraction with alcohol, followed by saponification and comparison in a Duboseq colorimeter. 30 gm. of peas, finely ground, were extracted with hot 95 per cent alcohol in a Soxhlet extractor for 6 hours. The extract was then saponified over night with 10 cc. of 10 per cent alcoholic potash which had been prepared with the usual precautions. After dilution with water the alcoholic soap solution was extracted by shaking repeatedly with ether. The ether was then volatilized, the yellow pigments taken up in 40 cc. of alcohol and quantitatively compared with one another in the Duboseq colorimeter. The results of this comparison are shown in Table I.

The analysis for relative vitamine concentration was made by the use of the rat as the experimental animal following the technique described in previous publications. Four animals were fed in each group, one group being used for each sample. The results as presented in Charts I and II show the complete data obtained with the various groups when fed peas at a 15 per cent level as the sole source of the fat-soluble vitamine in their diet.



TABLE I.

*Relative Yellow Pigment Content of Green and Yellow Peas.*

Color.	Designation.	Relative pigment values.
Yellow.	Commercial split.	39
"	Marrowfat.	44
"	Canadian field.	55
Green.	Small green.	76
"	Alaska.	100
"	Scotch beauty.	95

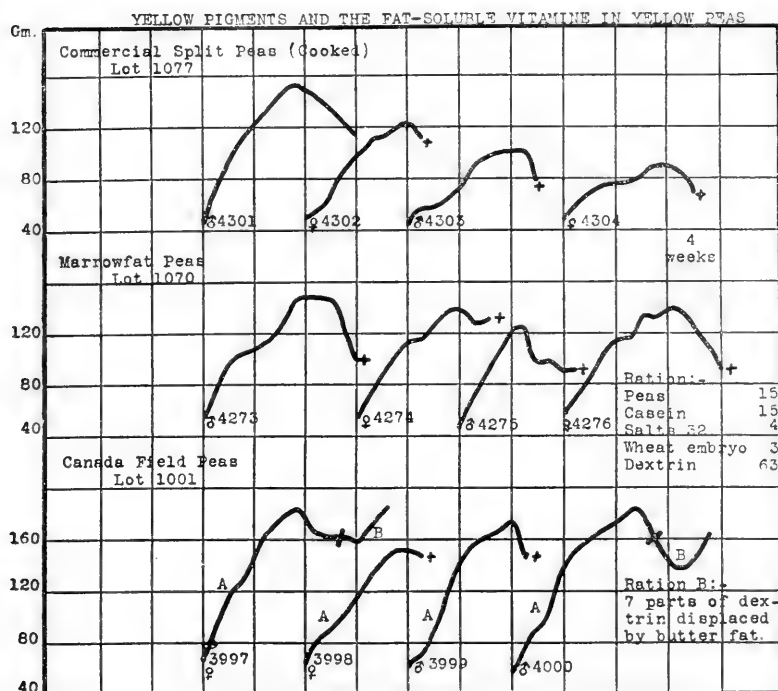


CHART 1.

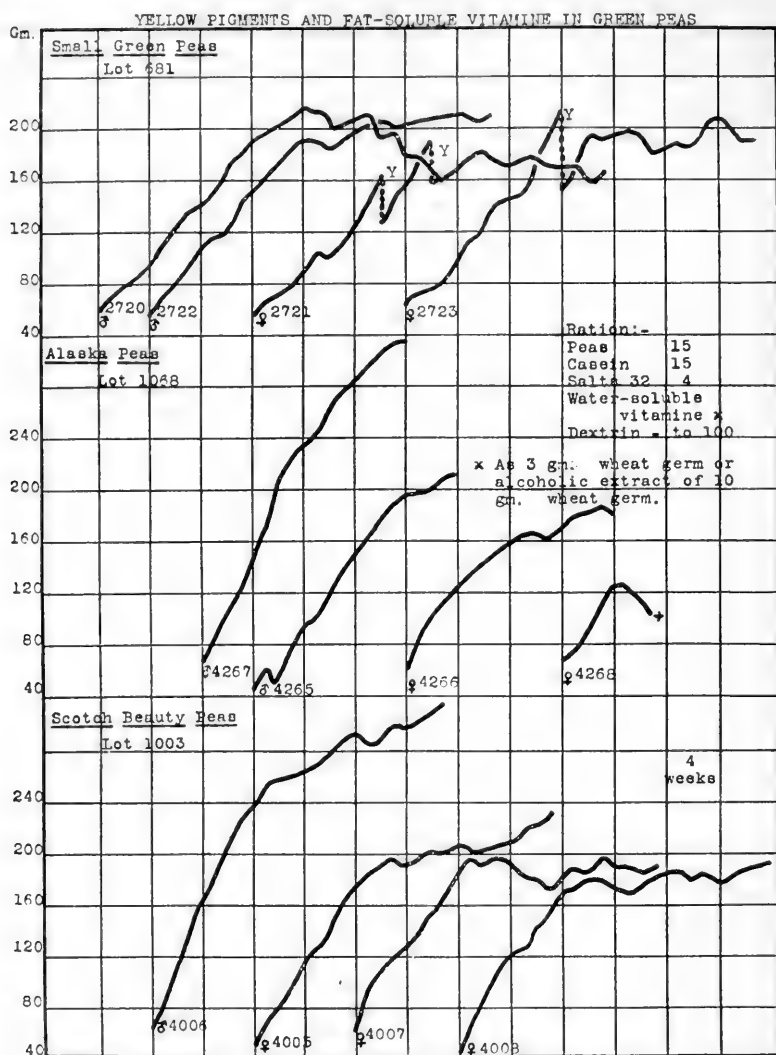


CHART 2.

In Chart I are shown the growth curves resulting when *yellow* peas were used as the medium for the introduction of the fat-soluble vitamine in the diet. In view of the abundance of hemi-cellulose found in peas it was surmised that the peas would have to be cooked to avoid causing digestive disturbances. This was done with the commercial split peas fed to Lot 1077 but not with any of the other samples as in the course of other feeding trials with peas it was found to be unnecessary although tympanites was occasionally observed. All the animals in the three lots contracted eye infections and four developed respiratory trouble in addition. In Lot 1001 two of the rats; *viz.*, Rats 3997 and 4000 responded gradually when butter fat was added to their ration. With the others no improvement of ration was attempted, yet from the consistency of the data as a whole no doubt is left that failure of growth and maintenance was due to a fat-soluble vitamine deficiency.

That yellow peas are not to be classed with seeds of very low fat-soluble vitamine concentration, such as wheat, oats, and barley, is shown by the very appreciable growth which occurred during the first 2 months on the experimental ration. On the basal ration without supplementation with peas young rats of the age used in these experiments rarely grow longer than 6 weeks.

Further experimentation with the amount of peas increased to 50 per cent of the ration gave more conclusive evidence of an appreciable fat-soluble vitamine content. On the marrowfat peas one rat with an initial weight of 60 gm. rose to 285 gm. after 4 months subsistence on the ration; yet its intake of the fat-soluble vitamine was not sufficient, as 4 weeks later, it as well as two others of this group, died from respiratory infections at an approximate weight of 170 gm.

The results of feeding green peas are shown in Chart II. Though fed at the same level as the yellow peas; *viz.*, 15 per cent, the curves of growth in themselves show their superiority as a source of the fat-soluble vitamine. In these three groups by way of contrast there was observed only one instance of suggested incipient ophthalmia and one instance of respiratory infections which occurred, respectively, in Rats 4267 and 4265. In the complete failure occurring with Rat 4268 the cause of death was obviously of a foreign nature as no symptoms indicating fat-soluble vitamine were observed.

## SUMMARY.

In ripe peas, out of six samples investigated, those of a green color, also carrying considerable yellow pigment, were far richer in their fat-soluble vitamine content than yellow peas which contained much less yellow pigment.

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## THE DETERMINATION OF CRESOL BY THE PHENOL REAGENT OF FOLIN AND DENIS.

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(Received for publication, May 9, 1921.)

At first sight the colorimetric method of Folin and Denis<sup>1</sup> promised to dispel most of the difficulties and uncertainties previously attending the determination of phenols in low concentrations. They and others immediately applied it to urine, feces, blood, etc., with apparently gratifying results. But a critical survey of the literature reveals that too little of the relatively unattractive foundation work has been executed. The temptation has been to utilize the method for the solution of certain problems without taking time for close scrutiny of its reliability under the operating conditions.

That a rather large number and wide variety of substances may develop color with the reagent has been shown by Gortner and Holm<sup>2</sup> and by Levine.<sup>3</sup> It may be doubted whether adequate measures have been taken to exclude non-phenolic substances in some reported work. But even if the color developed has been in all cases entirely due to "phenols," very little attention has been given to fundamental quantitative relations. For example, Folin and Denis determined the phenolic substances in urine as if they were exclusively phenol even though they state that "the most important phenol quantitatively is in fact paracresol."

The phenol reagent diluted with water yields a clear yellow solution. If there be added an excess of alkali or of an alkaline salt, even sodium acetate, the yellow color fades at a rate propor-

<sup>1</sup> Folin, O., and Denis, W., *J. Biol. Chem.*, 1915, xxii, 305, 309; 1916, xxvi, 507.

<sup>2</sup> Gortner, R. A., and Holm, G. E., *J. Am. Chem. Soc.*, 1920, xlii, 1678.

<sup>3</sup> Levine, V. C., *Science*, 1920, lii, 612.

tional to the degree of alkalinity. The final result is a colorless liquid, provided no traces of reducing impurities have gained access. Slightly alkaline mixtures remain clear; strongly alkaline ones soon deposit a considerable quantity of a white precipitate. The loss of yellow color marks the degradation of the active substance in the reagent, for at the end it no longer affords color with either phenol or sodium sulfite. If a phenol be present when the neutralizing agent is added the primary product of the reaction with the phenol is green. The green will change to blue at a rate proportional to the alkalinity and the temperature. The nature of the phenol is also of considerable influence. The primary products from *m*-cresol and *p*-cresol change color most readily, followed by that from phenol, while that from *o*-cresol lags far behind. The change in all cases is effected completely and quickly by sodium sulfite, as noted by Benedict and Theis.<sup>4</sup> Excessive alkali diminishes the intensity and permanence of the color, but in varying degree with different phenols.

If it is merely a matter of measuring a single phenol against the same phenol as a standard, no very strict regulation of conditions may be necessary, provided they be maintained closely parallel in sample and standard. But when a phenol or a mixture of phenols is to be measured against an entirely different standard phenol then it becomes important to select the set of conditions which will yield the most uniform and dependable results.

#### EXPERIMENTAL.

Assuming a certain appropriate weight of a given phenol in a certain final volume, it is necessary to determine (a) the proper quantity of reagent, (b) the proper nature, quantity, and reaction period of the alkali, (c) the effect of developing or stabilizing agents, and (d) the color factor for the phenol against a chosen standard phenol under the conditions finally selected as standard.

Stock solutions were prepared by weight from pure dry samples of phenol and the three cresols. These were freshly diluted for use and such aliquots were introduced into 100 cc. volumetric flasks as would yield colors closely approximating that yielded by 0.5 mg. of phenol under the same conditions of treatment,

<sup>4</sup> Benedict, S. R., and Theis, R. C., *J. Biol. Chem.*, 1918, xxxvi, 95.

which constituted the standard. All the work was done at room temperature, 22–29°C. Color comparisons were made in the Duboscq colorimeter, the standard being set at 20 and first read against itself as recommended by Folin and Denis.

Folin and Denis bettered their original reagent in accordance with a formula attributed to Bell. Wu<sup>5</sup> has made some further improvements, and his formula has been used in the present work. Preliminary experiments showed 3 cc. of reagent to be ample for the development of maximum color.

The alkali previously used has been sodium carbonate. As Experiment 1 will show, sodium bicarbonate yields heavier colors and more consistent factors and is sufficiently rapid in action. The mixture of each phenol with the reagent was diluted to 63 cc., 5 gm. of pure powdered  $\text{NaHCO}_3$  were added and the flask was swirled until solution was complete (about 2 minutes).

The colors were developed by the addition of 5 cc. of a 10 per cent solution of anhydrous sodium sulfite. Benedict and Theis merely state that sulfite is to be added "after the sodium carbonate." But since sulfite itself will reduce the reagent with production of a deep blue (Wu), it obviously must not be added until sufficient time has elapsed to insure complete inactivation of excess reagent. Experiments showed that in absence of phenol complete inactivation was effected by 5 gm. of  $\text{NaHCO}_3$  in 20 minutes. Therefore, all tests were allowed to stand one-half hour after solution of bicarbonate before sulfite was added. The contents of the flasks were then made to volume, mixed, and filtered, the first 25 cc. of the filtrate being discarded. Filtration may not be necessary under ordinary circumstances, for the only undissolved matter present appears to arise from traces of impurities in the reagent. But if filtration is omitted the flasks should be allowed to stand 15 minutes after addition of sulfite to insure sufficient time for its action. Colorimetric comparisons were executed immediately after filtration. The colors are probably fairly stable, but even so, the colorimetric comparison is so simple and rapid a process that a cogent excuse for delaying it is hardly likely to arise.

<sup>5</sup> Wu, H., *J. Biol. Chem.*, 1920, xliii, 189.

*Experiment 1.*—In comparison with the method above described as standard, tests were run in which 10 cc. of a solution of sodium carbonate of specific gravity 1.15 (16 gm. of  $\text{Na}_2\text{CO}_3$  per 100 cc.) were employed in place of sodium bicarbonate. The colors obtained were approximately the following fractions of the colors yielded by the standard method: Phenol, 0.90; *o*-cresol, 0.78; *m*-cresol, 0.83; *p*-cresol, 0.79. That is, a given weight of *o*-cresol, for example, yielded only 78 per cent as deep a color with sodium carbonate as it yielded with sodium bicarbonate. It is evident that the cresols are affected in different degree from phenol.

TABLE I.  
Factors Weight of cresol.  
Weight of phenol.

	Weight of <i>o</i> -cresol.	Weight of <i>m</i> -cresol.	Weight of <i>p</i> -cresol.	Corrected readings.	Average factor.			
	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>					
Series A. Temperature, 22- 24°. Phenol, 0.532 mg.	0.595	0.633	0.648	21.0	1.181			
				21.2				
				18.2	1.077			
				18.0				
		0.526	0.456	0.529	20.2	1.227		
					20.1			
Series B. Temperature, 28- 29°. Phenol, 0.435 mg. ....	0.526				19.6	1.193		
					19.9			
					20.7	1.101		
					21.3			
					19.6	1.207		
					20.1			
Final average factors.....	1.19	1.09	1.22					

*Experiment 2.*—In determining the color factor for each cresol by the standard method, two independent series of stock solutions were prepared, and two independent runs were made on each series. The results are reported in Table I. Each reading given is the average of at least three readings actually taken, and is corrected to be comparable to the standard phenol set at 20 on the same scale.

The theoretical factor  $\frac{\text{molecular weight cresol}}{\text{molecular weight phenol}}$  is 1.15. Taking commercial cresol as a 35:40:25 mixture of the isomers, the empirical factor would be 1.16.



*Experiment 3.*—To investigate the factor for xylenol, solutions were prepared from selected clean and colorless crystals of purchased material. Only one test was made. The factors were as follows: *o*-xylenol (1:2:4), 1.63; *p*-xylenol (1:4:2), 1.29. The theoretical molecular factor is 1.30.

It appears, therefore, that if the composition of the cresol be known to the extent that it is either predominantly a certain one of the isomers or the ordinary commercial mixture, and the proper indicated factor be employed, the results need not be in error by more than 1 part in 20.

The method has shown considerable practical value in this laboratory for the determination of phenolic preservatives in serums, etc. Whenever a sample of such a product has evinced abnormal physical or biologic characteristics it has here become customary to run a determination for its content of preservative. Elvove<sup>6</sup> distilled the highly diluted serum from a large flask to minimize interference by foaming, and applied Millon's reagent to the distillate. For a number of reasons the present phenol reagent is preferable to Millon's reagent. The writer has not attempted to dispense with distillation, for it has been necessary that the results reported should be free from suspicion. But it has been found that silicotungstic acid<sup>7</sup> is an effective precipitant for the substances which cause foaming, and therefore enables the distillation to be conducted more conveniently. To 1 cc. of serum in a 300 cc. flask are added 125 cc. of water, 4 cc. of 1:3 (by volume)  $H_2SO_4$ , 4 cc. of a 12 per cent solution of silicotungstic acid, and a fragment or two of hot pumice. The flask is connected to a nearly vertical condenser by a three-bend tube. The contents are slowly brought to boiling, and the distillate is received in a 200 cc. volumetric flask. When the latter is half full, *i. e.* when 100 cc. has been distilled off, the flame is withdrawn and 100 cc. more water are added to the distillation flask, then distillation is resumed and continued until the receiving flask is filled nearly to the mark. Of the distillate, made to the mark and mixed, either 20 or 50 cc., depending on whether phenol or cresol is the preservative probably used, are brought into a

<sup>6</sup> Elvove, E., *Bull. Hyg. Lab., U. S. P. H.*, No. 110, 1917, 25.

<sup>7</sup> This substance is now rather extensively used in alkaloidal work, particularly for the determination of nicotine, and should be procurable without difficulty.

100 cc. flask and the determination is executed according to the standard method previously given. Readings on the colorimeter should naturally be not too far from 20. Otherwise the more strongly colored solution should be properly diluted before comparison. Again, the latter procedure should be not trusted to too great an extent so that it may be necessary to repeat the determination on a more appropriate volume of distillate. If it is further necessary to distinguish between phenol and cresol the method previously described by the writer<sup>8</sup> may be utilized as a qualitative test. Into a test-tube are brought 10 cc. of the distillate, and in a similar tube a colorimetrically equivalent quantity of the standard phenol, diluted to 10 cc. To each tube are added 5 cc. of the special Millon reagent, and the tubes are heated as described. A comparison of the resulting colors in the tubes will plainly reveal whether the preservative employed has been essentially phenol or cresol.

#### SUMMARY.

It is shown that the quality and intensity of color developed by a phenol with the reagent of Folin and Denis is dependent both upon the nature of the phenol and the composition of the medium. The conditions affording colors of maximum intensity and most consistently comparable when developed from differing phenols have been determined. Accurate quantitative comparison between different phenols demands an empirical factor.

By use of prescribed empirical factors and adherence to standard working conditions, the cresols may be determined with fair accuracy as "total cresol."

The determination of phenolic preservative in serums is described.

<sup>8</sup> Chapin, R. M., *J. Ind. and Eng. Chem.*, 1920, xii, 771.

## THE EFFECT OF HYDROCHLORIC ACID INGESTION UPON THE COMPOSITION OF THE URINE IN MAN.

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(Received for publication, May 12, 1921.)

The data presented herewith are in a way an extension of an experiment reported in 1915 which concerned the effect of the administration of hydrochloric acid on the excretion of Na, K, Mg, and  $\text{NH}_3$  in the urine and feces of dogs (1). Increases in the amounts of all these substances were noted in the urine. The augmentation was especially noteworthy in the case of ammonia and almost negligible in the cases of calcium and magnesium. However, the data showed the calcium and magnesium content of the feces to be increased while sodium and potassium were unaffected.

Not long after the above results were published a paper by Givens (2) appeared in which the author concluded that his data failed to demonstrate any unequivocal effect of hydrochloric acid on the calcium and magnesium content of the feces of dogs. In view of the conflicting data it must be admitted that the question is still an open one but an examination of the respective sets of data will show that those of Givens show considerable lack of evenness.

In Table II on Diet B, Dog M does show a higher Ca excretion during the acid period than during the control period but instead of making the obvious interpretation that the acid was the cause Givens offers the explanation that "it seems more plausible to assume that in the fore period . . . the animal was trying to store up some lime after its depletion by the former low lime régime." "Former" refers to a previous experimental period. Neither is it at all obvious why, in the same table, the

excretion of Ca on the highest Ca diet falls short of the quantity excreted during the preceding acid period. This applies to the experiment with Dog N as well, but in this instance the Ca excretion during the period of highest Ca intake is even lower than that in a period of lower lime intake.

The problem as far as the dog is concerned has some rather difficult aspects. In order to overcome the difficulty resulting from the impossibility of separating the feces of the experimental periods sharply it is necessary to have long experimental periods. The diet must, of course, be uniform throughout the experiment and consequently of a nature so attractive that it will at no time be refused. As far as the dog is concerned this means that meat must be the principal constituent. Meat, however, is well suited to defeat the object desired since the ammonia resulting from its metabolism at once becomes available for the neutralization of any acid administered. Another possible difficulty presents itself in trying to determine where any bases excreted in response to acid administration will be found. It is not necessarily true that they need be in the feces corresponding to the day on which the acid was administered. Conceivably, they may be found in the feces of an earlier day depending upon what part of the alimentary tract is concerned with the excretion.

The present communication deals with the effect of hydrochloric acid ingestion upon the urinary composition in man. Advantage was taken of the experience gained in the earlier experiments and the diet so arranged as to make any variations as prominent as possible. It was ample calorically, but low in protein and sodium chloride. The components were rice, French fried potatoes, bananas, wheat bread, jam, butter, apple pie, and figs. None of the food was salted. The quantities were as uniform from day to day as careful measurement could make them and the only variation throughout the 7 days the diet was adhered to consisted in the ingestion of 3.65 gm. of hydrochloric acid in the form of tenth normal acid daily during the last 3 days. It was divided into three approximately equal portions and was drunk just before meals.

*Analytical Methods.*

For the estimation of Ca and Mg the procedure developed by McCrudden (3) was employed. Na and K were first isolated as the mixed sulfates and the potassium was then separated as the cobaltic nitrite compound. The yellow precipitate was then oxidized with a standard permanganate solution. The details of the process were those described by Drushel (4). Ammonia was

TABLE I.

Individual.	Day of diet.	Total N.	Chlorides as NaCl.	NH <sub>3</sub>	Na as NaCl.	K as KCl.	Ca	Mg	H <sub>3</sub> PO <sub>4</sub>	Ph
A. C. M.	3	6.85	4.22	0.294	3.56	4.61	0.058	0.104	1.11	6.70
	4	6.59	5.70	0.256	2.55	3.80	0.075	0.115	1.18	6.75
	5	7.95	10.47	0.559	5.19	8.74	0.090	0.181	1.64	6.28
	6	7.34	9.36	0.663	4.09	6.26	0.090	0.159	1.74	5.27
	7	6.97	11.69	0.731	4.11	7.17	0.113	0.161	1.69	5.05
R. L. S.	3	6.27	4.38	0.399	3.60	2.70	0.072	0.106	0.85	5.86
	4	6.16	5.03	0.300	3.70	4.10	0.100	0.094	0.97	6.44
	5	6.42	8.73	0.515	4.60	5.26	0.141	0.142	1.27	5.89
	6	6.29	9.76	0.730	4.29	5.96	0.123	0.135	1.53	5.05
	7	6.29	9.63	0.758	3.29	5.90	0.171	0.122	1.64	5.00

TABLE II.

	A. C. M.	R. L. S.
NaCl equivalent of increased NH <sub>3</sub> excretion.....	1.294	1.091
NaCl " " " Na " .....	1.41	0.41
NaCl " " " K " .....	2.49	1.81
NaCl " " " Ca " .....	0.090	0.172
NaCl " " " Mg " .....	0.282	0.161
NaCl " " " H <sup>+</sup> " .....	0.340	0.271
Total.....	5.906	3.915

determined by aeration, chlorides by the Volhard-Arnold method, and phosphates by titration with uranium acetate. The hydrogen ion concentration was measured electrometrically.

The effect of the acid on some of the urinary constituents was as given in Table I.

The sodium chloride equivalent of the 3.65 gm. of hydrochloric acid taken daily is 5.83 gm. If the sodium chloride equivalents

of the increased  $\text{NH}_3$ , Na, K, Ca, Mg, and  $\text{H}^+$  concentrations are calculated the results are as given in Table II.

The changes in H ion concentration are taken into account here for the following reason. The pH of the foreperiod shows that there must have been practically equal quantities of primary and secondary phosphates present. The pH of the acid period shows that practically all of the phosphate must have been present in the primary form. Consequently, while the conversion of the secondary phosphate into primary does not involve any change in the amount of phosphate excreted it does signify that a portion of the hydrochloric acid has been neutralized and must be taken into account in the attempt to account for the hydrochloric acid ingested.

If it be assumed that the increased phosphoric acid excretion is due to the conversion of disodium phosphate of the blood and tissues into monosodium phosphate, then for every atom of sodium excreted as sodium chloride which came from the disodium phosphate in question there will have been excreted an extra atom of sodium in the form of monosodium phosphate. Hence from the sum of the sodium chloride increases given above one atom of sodium for every extra molecule of phosphoric acid must be subtracted.

	A. C. M.	R. L. S.
NaCl equivalent of increased $\text{NH}_3$ , Na, K, Ca, Mg, and $\text{H}^+$ excretion .....	5.906	3.915
Na excreted as $\text{NaH}_2\text{PO}_4$ and not indicative of acid neutralization.....	0.328	0.340
NaCl equivalent of acid neutralizing factors.....	5.578	3.575

In the case of A. C. M. 96 per cent of the ingested acid is accounted for while in the case of R. L. S. the fate of only 61 per cent is apparent. Both of these percentages are undoubtedly influenced by physiological factors, the control of which was impossible, and it is probably true that the mean of the two would approach the true average value more closely than does either of them alone. This would leave about 22 per cent of the acid to be accounted for. The results of similar experiments on

the dog mentioned at the beginning indicate that increased excretion of calcium and magnesium by the intestine may be a factor, but no data are available in the present instance to indicate the extent to which this may be true.

If the bases of the body are drawn upon in any condition of acidosis in the same way that they have been drawn upon by the hydrochloric acid in the present experiments then it is interesting to see how the drain could be met to the best advantage. A simple calculation based upon the figures of A. C. M. in Table II shows that for every 3.65 gm. of hydrochloric acid (which is equivalent to 10.4 gm. of  $\beta$ -hydroxybutyric acid) there would be required in order to balance the increased  $\text{NH}_3$  formation and to meet the loss of K, Na, and  $\text{H}_3\text{PO}_4$ , 3.70 gm. of  $\text{KHCO}_3$ , 0.98 gm. of  $\text{K}_2\text{HPO}_4$ , and 3.88 gm. of  $\text{NaHCO}_3$ .

The calcium excretion *via* the urine is affected to a negligible extent but if the fecal excretion is affected in man to the same extent that it is in the dog as shown in the experiment previously referred to (1) then the above materials should be supplemented by a small quantity of some suitable calcium compound. It may be, however, that under conditions of alkali administration the effect on calcium excretion would be of no consequence.

#### CONCLUSIONS.

Data are presented which show that the ingestion of hydrochloric acid causes an increased excretion of potassium, sodium, ammonia, phosphoric acid, and hydrogen ions.

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# THE DISTRIBUTION OF CALCIUM AND PHOSPHORIC ACID IN THE BLOOD OF NORMAL CHILDREN.\*

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(Received for publication, May 3, 1921.)

The acid-base equilibrium of the body is a subject which has been much discussed, and concerning which many theories have been advanced. A review of the literature reveals a large volume of uncorrelated data, observations having been made on one or another constituent of the blood or urine under conditions which were in no way comparable. So far as we are aware no studies have been made on the distribution of calcium and the various compounds of phosphoric acid in corpuscles and plasma on the same sample of blood. It seemed to us that a knowledge of the relation of these substances to each other might throw some light on the mechanism of acid-base regulation which would be of value in certain pathological conditions. This paper is a report on a series of observations made on the blood of normal children ranging in age from 4 weeks to 14 years which we shall use as a basis of comparison for other work now in progress.

## *Technique.*

All the children under 3 years of age and a few of the older ones were from the wards of the University of California Hospital.<sup>1</sup> They were either surgical or convalescent cases and presumably had normal metabolism. The others were secured from a nearby orphanage and were apparently in good physical condition.

\* Part of the expense of this investigation was borne by a grant from the William H. Crocker fund for research in pediatrics.

<sup>1</sup> We wish to express our thanks to Dr. Bradford F. Dearing of the hospital staff for collecting the blood for us and for his interest and cooperation throughout this investigation.

*Collection of Blood.*—The blood of children over 18 months of age was collected before breakfast, about 15 hours after the last meal. That of children under 18 months was collected about 11 hours after the last feeding. 1 drop of a saturated solution of sodium citrate to every 5 cc. of blood was used to prevent coagulation. When possible, the blood was taken from the median arm vein. In the younger children it was usually taken from the external jugular, and in babies from the longitudinal sinus. From 25 to 30 cc. were drawn through a hollow needle into a clean, dry syringe, and of this, about 15 cc. were introduced by means of a short piece of glass tubing into a graduated centrifuge tube containing 3 drops of the citrate solution and 1 cc. of paraffin oil. Care was exercised to introduce the blood beneath the surface of the oil so as to avoid a possible exchange of acids and bases between the corpuscles and plasma. The remainder of the blood was quickly introduced into a test-tube containing 2 or 3 drops of the citrate solution and was used for the determinations on whole blood.

*Hematocrit Determinations.*—Immediately after the collection of the blood the plasma and corpuscles were separated by centrifugation at about 4,000 revolutions per minute for 20 minutes and the hematocrit reading taken. In order to secure accurate values it was found necessary to recalibrate all our centrifuge tubes. After removing the oil and plasma as completely as possible, the corpuscles were washed with 0.9 per cent NaCl solution and centrifugated as before. The volume of cells was again noted to insure a constant reading. Sundstroem and Bloor (1) suggest the use of a capillary tube for determining the percentage of cells, claiming that the readings taken in this way differ from those obtained in the graduated centrifuge tube. We found this to be true. Since, however, our hematocrit determinations were used primarily to check our work by computing the values in whole blood from those found in corpuscles and plasma, we necessarily used the readings of the centrifuge tube.

*Dilutions.*—In order to economize in material as well as time, we made dilutions which could be used for both the calcium and phosphoric acid determinations. The following were found satisfactory: (1) About 7 cc. of well mixed whole blood were laked with an equal volume of distilled water; (2) 3 cc. of corpuscles were laked with 9 cc. of distilled water; and (3) about 6 cc. of plasma were diluted with an equal volume of distilled water.

*Phosphoric Acid Determinations.*—Bloor's (2) nephelometric method with a few minor changes was used. Determinations were made on total, lipid, and inorganic phosphoric acids in whole blood, corpuscles, and plasma. So called "other forms" of phosphoric acid were calculated by subtracting the sum of the lipid and inorganic values from the total. The whole blood determinations were made merely as a check on our work, and in every case agreed with the calculated value within the limits of experimental error.

For the determination of lipid phosphoric acid, 3 cc. of each of the above dilutions of whole blood (1:1), corpuscles (1:3), and plasma (1:1) were used. The solutions were added drop by drop

to the alcohol-ether mixture in 25 cc. volumetric flasks and treated as directed by Bloor. Since the phosphoric acid content of the extract was twice as great as that of Bloor's, 5 cc. of the filtrate were used instead of 10 cc. In this way there was not only a large saving in alcohol and ether, but the evaporation of the smaller volume of liquid required much less time.

For the determination of inorganic phosphoric acid in whole blood, corpuscles, and plasma, 1 cc. of each of the dilutions as given above was added dropwise to 9 cc. of the acid ammonium sulfate solution and treated as directed.

For the determination of total phosphoric acid the dilutions were as follows: (1) 3 cc. of the 1:1 dilution of whole blood, made up to 10 cc.; (2) 1 cc. of the 1:3 dilution of corpuscles, made up to 5 cc.; and (3) plasma, 1:1 dilution.

1 cc. of each of the above dilutions was used. The solutions were introduced into Pyrex digestion tubes (1 by 10 inches) and evaporated to dryness on a water bath. This obviated the necessity of using glass beads during the digestion. Ordinary Pyrex tubes were found unsatisfactory, the strong acid mixture very quickly eating out the bottom and probably introducing an error into the determination. We succeeded in having satisfactory tubes made out of a special grade of Pyrex tubing. Instead of using the microburners with an improvised fume absorber as suggested by Bloor, we supported our tubes on wire gauze in the Kjeldahl apparatus where a large number of digestions could be carried on at one time.

We found it convenient to digest all the total and lipid phosphates (six tubes) at the same time and to carry them through the various steps together, using only one standard (0.006 mg.  $\text{H}_3\text{PO}_4$  per cc.), as a rule, for the nephelometric readings. For the inorganic phosphoric acid a standard containing 0.006 mg. of  $\text{H}_3\text{PO}_4$  was also used. In starting our work we experienced considerable difficulty on account of a precipitate which flocked out of the strychnine-molybdate solution when no phosphoric acid was present. This was found to be due to an insufficiency of strychnine sulfate for the amount of sodium molybdate used. No further difficulties were encountered and the remainder of the procedure was as given by Bloor. In order to secure the greatest accuracy possible we recalibrated all our pipettes and volumetric flasks

and used only one pipette of each volume throughout the series. In this way our actual and calculated values were in remarkably close agreement.

*Calcium Determinations.*—Lyman's (3) nephelometric method with a few modifications was used. 5 cc. of the whole blood (1:1), corpuscle (1:3), and plasma (1:1) dilutions were added drop by drop to 20 cc. of the trichloroacetic acid solution in 100 cc. Erlenmeyer flasks, filtered, and 10 cc. of each filtrate used for the determinations. If care is exercised, two 10 cc. portions can be obtained from each filtrate, enabling one to run duplicates if necessary. In using this method it is essential that the reactions be just right, else the calcium is not precipitated. To insure this we standardized our solutions and determined the end-points with utmost care, using alizarin rather than methyl orange for an indicator. Instead of shaking the solutions for 10 minutes as directed by Lyman, we allowed ours to stand in the refrigerator over night. By using sharply pointed centrifuge tubes and decanting the supernatant liquid quickly, we got better results than by pipetting the liquid from less pointed tubes. As was done in the case of phosphoric acid, the whole blood, corpuscle, and plasma solutions were carried through the various steps together. A standard containing 0.1 mg. of calcium was used, and was invariably checked against itself before the readings on the unknown solutions were taken.

In order to determine the accuracy of the method, known quantities of calcium were added to blood and blank solutions, and in all cases were recovered quantitatively. In addition, many determinations which seemed extraordinarily high were checked by means of a potassium permanganate titration method, the principle of which was essentially the same as that of Lyman's except that the precipitate of calcium oxalate was dissolved in  $\times$  sulfuric acid and titrated with 0.01  $\times$  permanganate solution. After making determinations on about twenty samples of blood with both methods, we finally adopted Lyman's, since, with the titration method, our whole blood, corpuscle, and plasma readings frequently failed to check, while with the latter, our actual and calculated values practically always agreed within the limits of experimental error. We attribute the lack of agreement between the results secured with the former method to the fact that the end-point with 0.01  $\times$  potassium permanganate is very difficult

to obtain, and 1 drop too much or too little may introduce an error of 20 per cent in the final value, the percentage of course, depending upon the volume of permanganate used in the titration.

*Alkali Reserve.*—The  $\text{CO}_2$ -combining power of plasma was determined by the Van Slyke-Cullen (4) method without change.

The results of the analyses are given in Tables I and II and are expressed in mg. of calcium and phosphoric acid per 100 cc. of whole blood, corpuscles, and plasma. Certain values which seemed exceptionally high were not included in the average. To be consistent, if the value of a constituent was not included in the average for corpuscles, the values of that same constituent were omitted from the averages for whole blood and plasma.

#### DISCUSSION.

The distribution of calcium and the various compounds of phosphoric acid was studied in thirty-four normal children, seventeen boys and seventeen girls. In general, the values for phosphoric acid are comparable to those reported by Bloor (5) and others. The total phosphoric acid values of corpuscles averaged 257 mg. per 100 cc. in boys, and 255 mg. in girls as compared with Bloor's values of 248 mg. for men and 249 mg. for women. On the whole, the averages for boys tend to run higher than those for girls. This is especially striking in the lipid phosphoric acid content of the corpuscles, the average for boys being 65.7 mg. per 100 cc. with variations from 36 to 84, and for girls, 55.8 mg. with variations from 33 to 72. Bloor's averages were 57.0 and 56.6 mg. for men and women, respectively. Another striking difference between the values reported for adults and children is found in the inorganic phosphoric acid content of corpuscles. For men, Bloor found an average of 18.7 mg. per 100 cc. and for women, 15.7 mg. Our values for boys ranged from 6.5 to 20.9 with an average of 12.1, while those for girls varied from 5.7 to 26.0 mg. with an average of 10.3. The inorganic phosphoric acid content shows a greater percentage variation than that of any phosphorus compound in the corpuscles, or approximately 221 per cent in boys and 356 per cent in girls.

In the plasma the higher values of the total and lipid phosphoric acids are again noted in the boys. The total phosphoric acid content varied from 33 to 48 mg. in boys with an average of

TABLE I.  
The Distribution of Calcium and Phosphoric Acid Compounds in the Blood of Normal Boys.

No.	Age.	Name.	H <sub>2</sub> PO <sub>4</sub> per 100 cc.										Calcium per 100 cc.										CO <sub>2</sub>	Red since blood last meal.								
			Whole blood.				Corpuscles.				Plasma.				Other forms.				Whole blood.						Corpuscles.				Plasma.			
			Total.	Li- poid.	Inor- ganic.	Other forms.	Total.	Li- poid.	Inor- ganic.	Other forms.	Total.	Li- poid.	Inor- ganic.	Other forms.	Total.	Li- poid.	Inor- ganic.	Other forms.	Whole blood.	Corpus- cles.	Plasma.	Vol. per cent			hrs.							
1	4 wks.	B. G.	118.0	41.0	11.5	65.5	258.0	72.0	9.0	177.0	33.0	20.0	13.0	0.0	9.0	9.4	51.5	38.2	11													
2	2 mos.	B. B.	127.2	45.0	9.2	73.0	324.0	74.0	12.6	237.4	43.2	35.5	8.1	-0.4	-0.4	11.0*	46.2	30.4	11													
3	7 "	B. S.	100.0	40.0	8.4	51.6	240.0	73.0	8.1	158.9	36.0	28.5	8.4	-0.9	9.9*	14.0*	8.4	39.5	30.1	11												
4	3 yrs.	B.	128.0	50.0	9.0	69.0	279.0	72.0	11.2	195.8	45.0	37.0	7.4	0.6	11.6	11.2	11.8	50.0	36.5	15												
5	4 "	W. S.	110.0	31.0	10.8	68.2	234.0	36.0	9.7	188.3	42.0	30.0	11.4	0.6	5.6	5.6	5.5	48.6	36.2	15												
6	5 "	E. S.	150.0	50.0	9.2	90.8	300.0	74.0	9.2	216.8	45.6	35.0	10.0	0.6	12.4	12.0	12.4	59.3	41.4	15												
7	6 "	D. K.	138.0	50.0	14.2	73.8	264.0	72.0	18.9	153.1	46.2	36.0	10.8	-0.6	11.4	11.6	11.0	50.4	42.3	15												
8	6 "	J. S.	172.0*	57.0*	13.0	102.0*	360.0*	90.0*	19.6	250.4*	54.0*	45.0*	9.0	0.0	10.0	12.0	8.6	51.8	40.0	15												
9	6 "	V. B.	115.0	37.0	15.0*	63.0*	240.0	55.0	31.6*	153.4*	36.0	30.0	5.0*	1.0	1.0	12.0	48.5	39.5	15													
10	7 "	M. B.	110.0	37.5	16.3*	56.2*	231.0	60.0	30.4*	140.6*	30.6	23.5	7.2*	-0.1	-0.1	12.0	48.5	39.5	15													
11	8 "	A. G.	125.0	42.4	9.1	73.5	240.0	58.0	7.8	174.2	40.2	31.0	9.2	0.0	0.0	6.4	9.6	63.8	42.3	15												
12	9 "	H. B.	130.0	47.0	11.0	72.0	270.0	64.0	17.0	189.0	45.0	37.0	7.4	0.6	8.0	6.4	9.6	51.5	38.5	15												
13	9 "	W. H.	138.0	52.0	14.0	74.0	252.0	70.0	14.2	167.8	48.0	35.0	14.0	-0.1	-0.1	10.4	10.4	55.1	44.0	15												
14	11 "	E. J.	88.0*	41.5	12.4	34.1*	150.0*	40.4	10.0	99.6*	52.8*	40.0	12.2	0.6	8.8	5.8	10.4	52.5	36.1	15												
15	12 "	R. A.	114.0	50.3	14.0	49.7	240.0	84.0	20.9	135.1	43.5	33.0	10.8	-0.3	9.2	10.0	9.4	35.9	35.9	15												
16	12 "	W. J.	100.0	48.6	7.0	44.4	189.0	71.0	6.5	111.5	43.8	35.0	7.4	1.4	8.4*	5.8*	11.0*	47.6	38.1	15												
17	13 "	C. R.	147.0	50.5	7.3	89.2	300.0	76.0	6.5	217.5	39.0	31.0	7.9	0.1	10.1	5.2	14.4	46.6	41.5	15												
Average.....			123.3	44.5	10.6	68.8	257.4	65.7	12.1	178.6	41.1	32.3	9.8		9.4	9.0	10.2	50.8	38.2													

\* Not included in the average.

TABLE II.

## The Distribution of Calcium and Phosphoric Acid Compounds in the Blood of Normal Girls.

No.	Age.	Name.	H <sub>3</sub> PO <sub>4</sub> per 100 cc.												Calcium per 100 cc.						CO <sub>2</sub>	Red blood cells.	Time since last meal.					
			Whole blood.				Corpuscles.				Plasma.				Whole blood.			Corpuscles.						Plasma.				
			Total.	Li- poid.	Inor- ganic.	Or- ganic.	Total.	Li- poid.	Inor- ganic.	Or- ganic.	Total.	Li- poid.	Inor- ganic.	Or- ganic.	mg.	mg.	mg.	mg.	mg.	mg.				mg.	mg.	Vol.	per cent	hrs.
1	6 mos.	B. S.	100.0	32.5			234.0	48.0					33.0	24.8	8.1	0.1	9.3	5.2	11.0	47.7	33.1	11						
2	3 yrs.	A. N.	136.3	40.0	5.8	90.5	330.0	72.0	5.7	252.3	31.5	26.0	6.0	-0.5	11.3						51.8	35.5	15					
3	4 "	J. S.	110.0	32.4	6.7	70.9	225.0	50.0	6.6	168.4	34.5	27.0	6.7	0.8							59.5	39.5	15					
4	4 "	E. B.	116.0	37.0	9.2	69.8	240.0	52.0	9.6	178.4	37.2	28.0	9.6	-0.4	10.0	10.0	10.0	10.0	49.2	38.4	15							
5	4 "	M. K.	105.0	40.8	10.0	54.2	228.0	66.0	11.6	150.4	36.0	27.0	9.0	0.0	9.1	8.4	9.6	49.2	35.6	15								
6	7 "	E. C.	150.0	46.0	11.6	92.4	300.0	65.0	13.0	222.0	44.0	33.0	9.5	1.5						59.3	41.2	15						
7	7 "	R. M.	122.0	37.0	14.0	71.0	252.0	58.0	26.0	168.0	36.0	26.0	8.0	2.0	9.2	8.0	10.0	48.6	40.5	15								
8	7 "	G. A.	144.0	43.0	11.1	89.9	294.0	68.0	9.8	216.2	37.2	25.0	12.2	0.0	7.8	5.7	9.2	48.6	41.6	15								
9	7 "	L. P.	116.2	38.5	9.3	68.4	230.0	54.4	8.5	167.1	40.0	28.8	10.3	0.9						54.3	38.0	15						
10	8 "	T. P.	90.0	26.0	8.1	55.9	177.0	33.0	10.8	133.2	27.1	20.0	6.3	0.8	8.0	5.2	10.0	50.5	41.1	15								
11	10 "	E. G.	142.0	48.0	13.3	80.7	279.0	63.0	10.4	205.6	55.5	40.0	15.0	0.5						55.0	41.4	15						
12	10 "	V. N.	132.8	46.0	7.2	79.6	270.0	61.0	5.0	204.0	42.0	35.0	9.0	-2.0	10.0	10.4	9.7	59.5	40.3	15								
13	11 "	V. R.	132.0	40.5	10.0	81.5	270.0	60.0	14.2	195.8	36.0	29.0	6.6	0.4	9.8	11.0	9.0	58.1	41.3	15								
14	12 "	J. N.		51.5	10.0			71.0	9.9		47.8	37.5	10.3	0.0	8.3					42.7	15							
15	12 "	E. C.	122.0	35.0	7.2	79.8	258.0	55.0	9.0	194.0	27.0	21.5	6.3	-0.8	9.1	9.6	8.8	56.2	41.2	15								
16	13 "	L. P.	126.0	34.0	7.5	84.5	276.0	51.5	7.2	217.3	33.0	25.0	8.1	-0.1	10.2	10.2	10.0	47.9	37.7	15								
17	13 "	E. M.	95.0	25.0	7.5	62.5	219.0	40.0	6.7	172.3	24.6	17.0	8.1	-0.5	9.8	9.4	10.0	49.8	37.4	15								
Average.....			121.2	38.4	9.3	75.4	255.1	55.8	10.3	189.6	36.6	27.7	8.8		9.4	8.5	9.8	52.8	39.2									
General average for children.....			122.2	41.3	9.9	72.3	256.2	60.6	11.1	184.5	38.7	29.9	9.3		9.4	8.7	10.0	51.8	38.7									

41.1, while in girls the range was from 27 to 47 mg., the average being 36.6, as contrasted with Bloor's values of 32 and 36.2 mg. for men and women, respectively. In boys, the lipid phosphoric acid content of plasma ranged from 20 to 40 mg., the average being 32 or approximately 50 per cent higher than Bloor found in men. A possible explanation of the higher values in boys than in girls or adults might be found in their greater muscular activity. The difference between the values found in girls and women is not so marked. For the former, the average was 27.7 mg. with variations from 20 to 37.5, and for the latter, 24.9 mg. The inorganic phosphoric acid values of plasma were approximately the same in boys and girls, being 9.8 and 8.8 mg. per 100 cc., respectively. The variations in the boys did not equal 100 per cent and those in the girls did not exceed 150 per cent which is in striking contrast to the percentage variation of the inorganic phosphoric acid content of the corpuscles. Marriott and Howland (6) using a different method, found the inorganic phosphorus content of the serum to vary from 1 to 3.5 mg. (approximately 3.2 to 11.2 mg.  $\text{H}_3\text{PO}_4$ ) per 100 cc. in normal infants, and Denis and Minot (7) using Bloor's method found a similar range in adults suffering from various diseases other than nephritis and cardiorenal conditions. It should be noted that the sum of the inorganic and lipid phosphoric acid values in plasma is equal to the total. Bloor found a small amount of an unknown phosphoric acid compound (up to 10 per cent of the total) in plasma. This value was obtained by subtracting that of the inorganic phosphoric acid from the acid-soluble. We did not make determinations on the acid-soluble fraction, but as the sum of the inorganic and lipid phosphoric acid values is equal to the total, within the limits of experimental error, the presence of this substance in an appreciable quantity seems doubtful. However, in certain pathological conditions which we are now investigating, we have found considerable quantities of the so called "organic" phosphorus. In many cases it is interesting to note the approximate equilibrium of the inorganic phosphoric acid between corpuscles and plasma. This is contrary to Bloor's findings in men and women which show a much higher concentration in the corpuscles than in the plasma. We are not able to explain the significance of the differences between boys and girls and between children and adults.



Until recently, it has generally been conceded that little or no calcium exists in the corpuscles. Howland and Marriott (8) found about half as much calcium in normal whole blood as in serum, from which they concluded that there was no calcium in the corpuscles and advocated the use of serum or plasma for calcium determinations. Many references to their work have been found in the literature. Hammarsten (9) cites the work of Gyns, Köppe, Hamburger, and others showing that blood cells are impermeable to calcium and magnesium, although Schmidt (10) in 1850 claimed that corpuscles contain a considerable amount of this metal. Recent investigations of Cowie and Calhoun (11) and others have confirmed Schmidt's contention by showing that calcium is present in corpuscles in appreciable quantities. Cowie and Calhoun used Lyman's method and made many determinations on a few cases, the values in one man being as follows: whole blood, 8.9 mg.; corpuscles, 4.26 mg.; and plasma, 12.07 mg. per 100 cc. We were not able to ascertain whether the determinations were made on the same or different samples of blood. Brown, MacLachlan, and Simpson (12) also using Lyman's method found an average of 9.5 mg. of calcium per 100 cc. of whole blood in eighteen normal infants, which is in close agreement with our value of 9.4 mg. Lyman reported an average of 6.1 mg. per 100 cc. of whole blood for men and 7.1 mg. for women. One woman had a value of 9.4 mg. The great majority of investigators have used serum or plasma for their determinations, the reported values of 9 to 11 mg. agreeing well with our average of 10 mg. per 100 cc. of plasma. The most striking feature of this investigation is the large amount of calcium found in the corpuscles, in many cases the content being equal to or greater than that in the plasma. As was found in the case of inorganic phosphoric acid, the greatest percentage variation of the calcium content occurred in the corpuscles, the boys showing wider limits than the girls. One boy, age 4 years, had an extremely low concentration in the blood, his values being: whole blood, 5.6 mg.; corpuscles, 5.6 mg.; and plasma, 5.5 mg. per 100 cc. His phosphoric acid values were well within the normal range. Neither age (within the above range) nor sex appears to be a factor in the calcium concentration of the blood or its distribution between plasma and corpuscles. Meigs, Blatherwick, and Cary (13) showed that in heifers the calcium content of plasma

tends to become lower with advancing age up to 6 months. In some studies on the new-born now in progress, we have found the average calcium values of five babies under 14 hours of age to be as follows: whole blood, 8.7 mg.; corpuscles, 5.9 mg.; and plasma, 12.6 mg. per 100 cc. While we have not yet sufficient data from which we can draw conclusions, it appears that the calcium content of plasma is higher at birth than in later life, the average value for corpuscles being markedly less than that in older children.

The plasma of thirty-two children had an average  $\text{CO}_2$ -combining power of 51.8 volumes per cent. Sawyer, Stevens, and Bauman (14) report a plasma carbonate value of 54 volumes per cent in children between the ages of 4 and 8 years. Schloss and Stetson (15) found a range of from 46.1 to 76.1 volumes per cent in normal infants. Only one of our values (39.5) was less than Schloss and Stetson's lower limit, and we consider this too low to be within the normal range. It is interesting to note that in this case the calcium content of plasma is lower than the average and that of the corpuscles is considerably higher.

#### SUMMARY.

The alkali reserve of plasma and the distribution of calcium and the various compounds of phosphoric acid in the blood were studied in thirty-four normal children whose ages ranged from 4 weeks to 14 years.

From the above data it appears that the blood corpuscles are richer in all types of phosphoric acid compounds than plasma. The amount of unknown phosphoric acid in plasma is negligible, if any, while in corpuscles it averages approximately 70 per cent of the total.

In general, the values for boys averaged slightly higher than those for girls. The lipid phosphoric acid content of corpuscles averaged 17.7 per cent higher in boys than in girls, while the plasma value in boys was 16.6 per cent higher than that in girls. The inorganic phosphoric acid content of corpuscles showed the greatest percentage variation of all the phosphorus compounds of the blood.

The average calcium content of corpuscles was found to be slightly less than that of the plasma, the values in mg. per 100 cc. being as follows: whole blood, 9.4 mg.; corpuscles, 8.7 mg.; plasma, 10.0 mg. A relation between the calcium and phosphoric acid contents of the blood is not apparent.

The CO<sub>2</sub>-combining power of the plasma averaged 51.8 volumes per cent in thirty-two children. No relation between the alkali reserve and the concentration of calcium and phosphoric acid in the blood can be established.

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## STUDIES OF AUTOLYSIS.

### VII. AUTOLYSIS OF BRAIN.

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(Received for publication, May 10, 1921.)

Brain undergoes autolytic decomposition very slightly when measured by amino-acid production and when compared with most other tissues. The evidence of the reaction even under the most favorable conditions is so slight that it may easily be overlooked, or be incorrectly interpreted. It is because of this that the brain proteins are assumed (1) to be peculiarly resistant to autolytic changes and not subject to those rather rapid fluctuations of mass which occur for example in the liver, and in other organs. The peculiar stability of brain and nerve proteins thus appears to relate itself to those functions of the brain which seem to demand a peculiarly stable physical structure, the functions of memory, habit, instinct, etc.

While there can be no question about the stability of the protein framework of the brain cells, it does not necessarily follow that the autolytic mechanism is wanting, or is strikingly different from that of other organs. The outstanding fact of stability and permanence of brain cell structure may equally well be explained by the peculiarly perfect protection against local acidosis which the large blood supply of the brain insures. The respiratory center is itself nerve tissue. It is characterized by its sensitiveness to H ion changes, to  $\text{CO}_2$  and O content (2-8). By its regulation the blood always supplies the brain with oxygen sufficient to prevent asphyxial reactions from developing, except under pathological conditions. With abundance of oxygen supplied, there is also very perfect  $\text{CO}_2$  elimination, and a supply of buffer salts which tends to prevent the development of local acidosis. In short, the regulation of  $\text{CO}_2$  elimination, of constant H ion level, and of oxygen supplied the body is very largely effected by a portion of the central nervous system itself. Conditions giving rise to acido-

sis of the liver, a group of muscles, or the kidney may be without influence on the brain, or on tissue respiration as a whole. On the other hand any condition of the brain tending toward acidosis, asphyxia, faulty  $\text{CO}_2$  elimination, or rising H ion concentration, will result in an immediate respiratory and circulatory response affecting the entire body. The brain may be said to protect itself from asphyxia and acidosis, and incidentally in so doing to protect the rest of the body. If the brain tissue is like other tissues it will not exhibit autolytic changes as long as it is so perfectly protected against the conditions under which alone autolysis can go on.

Clinically we have much evidence to support the idea that atrophic changes do go on in the brain, just as they do in other tissues. We have the familiar losses of consciousness in brain anemias or asphyxias, rapidly progressing to more permanent functional losses and death where the condition is at all prolonged. General acidosis frequently leads to coma from which no measure of relief can save the patient, suggesting a progressive degenerative change in the central nervous system of an irreversible character. Local pressures in the brain lead to paralyses, and these may become permanent as a result of irreversible atrophic changes. The pathologist recognizes and correlates functional losses with brain or nerve degeneration. These degenerations are perfectly analogous to the atrophies of other tissues. They suggest that under favorable conditions autolysis may proceed quite rapidly, and in the delicate and essential protein structures of the brain cells produce irreversible losses and obliterations of a framework upon which the unique characteristics of the central nervous tissue depend.

It appeared worth while, therefore, to review and repeat such significant work as has been reported on brain autolysis and to compare the autolytic reaction of brain with that of other tissues. Levene and Stookey (9) showed in 1903 that brain autolysis produces such characteristic protein decomposition products as peptones, amino compounds, and free  $\text{NH}_3$ , while the coagulable protein fraction diminishes. They found that acids accelerate this process, while alkalies inhibit it. Their experiments were few, however, and confined to the demonstration of the fact of brain autolysis, which had not previously been convincingly shown.

## EXPERIMENTAL PART.

The experimental technique is exactly like that described in previous papers (10). 50 gm. of brain tissue, finely ground, are made up to 250 cc. with water and toluene. 25 cc. samples are taken, made up to 100 cc. with trichloroacetic acid to precipitate the proteins and 25 cc. aliquots of the filtrates titrated by the formol method for amino-acids. The titration figures are small and the errors due to end-points are proportionately large. By matching end-point colors with a standard and by using small burettes graduated in 0.01 cc., the titration errors have been kept as low as possible and the figures, though actually small, are nevertheless characteristic and significant.

A few typical examples, selected from the large number of experiments performed, are presented.

From Tables I to V and Fig. 1 we are able to conclude that brain pulp autolyzes normally as do other tissues *in vitro*. The

TABLE I.  
*Sheep Brain.*

No.	Condition.	0.20 N amino-acids.					Net gain in 33 days.
		Days.					
		0	2	3	10	33	
		cc.	cc.	cc.	cc.	cc.	cc.
I	Control.....	0.40	0.55	0.65	0.85	1.25	0.85
II	“ in 0.01 N HCl.....	0.40	0.55	0.68	1.00	1.50	1.10
III	“ “ 0.02 N HCl.....	0.40	0.57	0.65	1.15	1.45	1.05
IV	“ “ 0.04 N HCl.....	0.40	0.50	0.65	0.70	0.90	0.50
V	“ + 3 gm. Na <sub>2</sub> HPO <sub>4</sub> .....	0.40	0.55	0.60	0.80	1.05	0.65

TABLE II.  
*Calf Brain.*

No.	Condition.	0.20 N amino-acids.				Net gain in 30 days.
		Days.				
		0	1	5	30	
		cc.	cc.	cc.	cc.	cc.
I	Control.....	0.25	0.35	0.60	0.60	0.35
II	“ in 0.01 N HCl.....	0.25	0.40	0.90	1.40	1.15
III	“ “ 0.02 N HCl.....	0.25	0.40	0.80	1.30	1.05
IV	“ “ 0.01 N NaOH.....	0.25	0.25	0.35	0.45	0.20
V	“ + 5 gm. CaCO <sub>3</sub> .....	0.25	0.40	0.45	0.60	0.35

TABLE III.  
*Calves Brains.*

No.	Condition.	0.20 N amino-acids.						Net gain in 30 days.		
		Days.								
		0	1	2	5	10	20	30	10	30
		cc.	cc.	cc.	cc.	cc.	cc.	cc.	cc.	cc.
I	Control.....	0.3	0.35	0.37	0.40	0.45	0.50	0.55	0.20	0.25
II	“ in 0.005 N HCl..	0.3	0.4	0.55	0.65	0.80	0.90	1.00	0.60	0.70
III	“ “ 0.01 N HCl..	0.3	0.4	0.60	0.65	1.00	1.05	1.40	0.80	1.10
IV	“ “ 0.02 N HCl..	0.3	0.4	0.70	0.75	1.00	1.40	1.60	1.10	1.30
V	“ “ 0.04 N HCl..	0.3	0.25	0.30	0.33	0.40	0.40	0.40	0.10	0.10
VI	“ “ 0.1 N HCl..	0.3	0.25	0.30	0.30	0.35	0.45	0.55	0.15	0.25
VII	“ “ NaHCO <sub>3</sub> .....	0.3	0.35	0.35	0.35	0.40	0.45	0.50	0.15	0.20
VIII	“ “ K <sub>2</sub> HPO <sub>4</sub> .....	0.3	0.35	0.35	0.35	0.40	0.40	0.50	0.10	0.20

TABLE IV.  
*Calves Brains.*

No.	Condition.	0.20 N amino-acids.				Net gain in 15 days.
		Days.				
		0	1	3	15	
		cc.	cc.	cc.	cc.	cc.
I	Control.....	0.25	0.40	0.50	0.50	0.25
II	“ in 0.04 N HCl.....	0.25	0.60	0.55	0.60	0.45
III	“ “ 0.02 N HCl.....	0.25	0.50	0.70	0.80	0.65
IV	“ + 1 gm. K <sub>2</sub> HPO <sub>4</sub> .....	0.25	0.40	0.50	0.45	0.20
V	“ + 2 “ K <sub>2</sub> HPO <sub>4</sub> .....	0.25	0.40	0.40	0.40	0.15
VI	“ + 5 “ K <sub>2</sub> HPO <sub>4</sub> .....	0.25	0.35	0.30	0.30	0.05
VII	“ + 10 “ K <sub>2</sub> HPO <sub>4</sub> .....	0.25	0.40	0.30	0.35	0.10

TABLE V.  
*Sheep Brain, Foreign Protein, and Acid.*

No.	Condition.	0.20 N amino-acids.					Net gain.	Digestion of foreign protein.
		Days.						
		0	1	3	7	15		
		cc.	cc.	cc.	cc.	cc.	cc.	
I	Control.....	0.25	0.50	0.50	0.60	0.60	0.35	
II	“ in 0.04 N acid....	0.25	0.60	0.65	0.65	0.70	0.45	
III	“ “ peptone.....	0.75	1.90	2.10	2.30	2.40	1.65	1.25
IV	“ “ “ acid..	0.75	1.40	1.30	1.50	1.70	0.95	0.50
V	“ “ gelatin.....	0.40	0.70	0.90	1.00	1.10	0.70	0.25
VI	“ “ “ acid..	0.40	1.20	1.50	1.60	1.90	1.50	1.05



amount of protein in brain is small, approximately 7 per cent of the total, while in muscle it approximates 18 to 20 per cent, and in liver 15 per cent or more. It is not surprising, therefore, that the figures for brain autolysis should be much smaller than those for liver, muscle, and other tissues. The addition of acid increases the speed and extent of autolysis of brain, but the amount required for maximum digestion is smaller than for tissues like the liver.

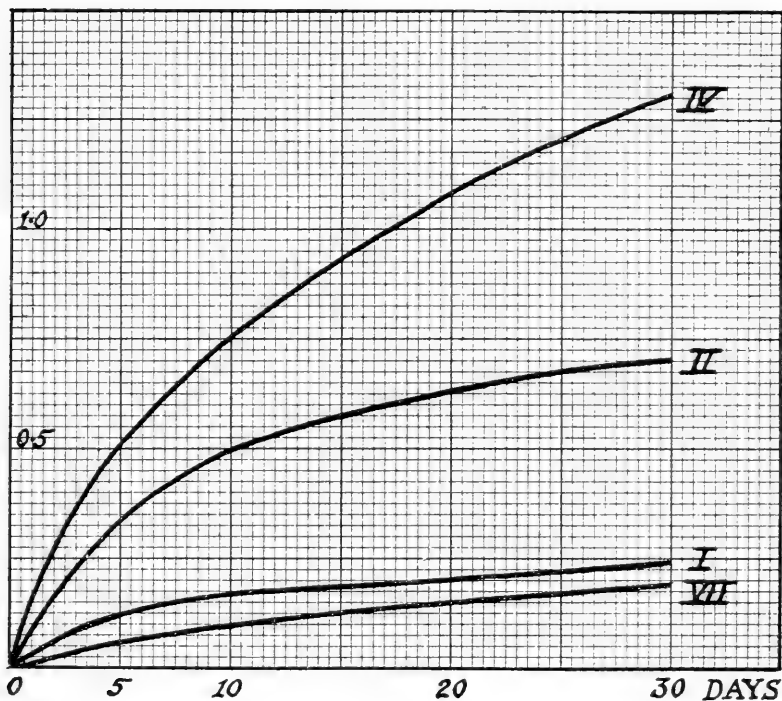


FIG. 1.

This is correlated evidently with the protein content, the more protein present the more acid can be added before the H ion rises to the point of inhibiting the reaction. With liver, sufficient acid to make the digesting mixture 0.04 to 0.1  $\times$  gives maximum autolysis. With brain an acidity of 0.02  $\times$  is optimum.

Foreign proteins such as gelatin and peptone, which are digested by the autolytic enzymes of the liver, are also found to digest in

the brain mixtures. It is of interest to note that peptone digests better in the nearly neutral control than it does in the rather strongly acid brei used, while gelatin digests much better in the acid medium.

The stability of brain structure depends not on any lack of proteolytic enzymes in the cells, but upon the fact that normally the brain proteins are not available substrata for the enzymes. They become available under the conditions that make liver proteins available for autolytic hydrolysis; namely, acidosis within the cells. That local acidoses do not frequently occur in brain tissue in spite of its large  $\text{CO}_2$  production, is due we believe to the exceptionally large blood supply to that organ, and to its ability to modify the respiratory and circulatory rate so as to prevent any accumulation of  $\text{CO}_2$  or other acid. By its extreme sensitiveness to increased H ion concentration and  $\text{CO}_2$  and by its position as master tissue of the body it automatically prevents just those conditions from arising within itself which would eventuate in its own autolytic disintegration.

#### SUMMARY.

1. Brain tissue autolyzes in the same way as other tissues thus far examined though quantitatively on a smaller scale comparable to the low protein content of brain tissue.

2. The speed and extent of the proteolysis is determined by the H ion concentration of the mixture. In alkaline or neutral media autolysis is inhibited. It is increased in proportion to the acid added.

3. The optimum acidity for autolysis of brain tissue is about 0.02 N, or much less than that for liver, kidney, and other epithelial tissues. The small amount of acid required corresponds to the small amount of protein present and made available for digestion by the addition of acid.

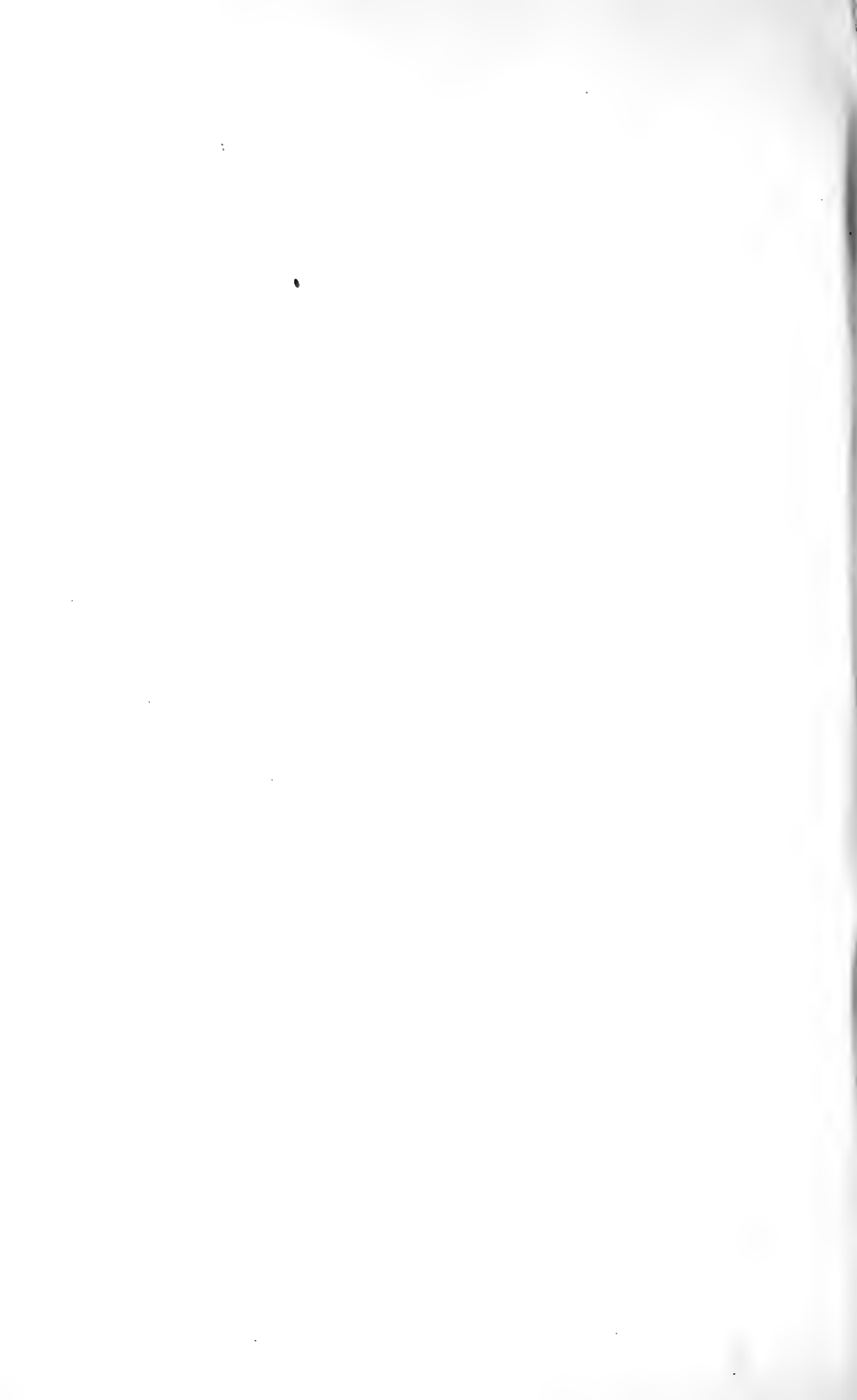
4. Brain cells contain proteolytic enzymes which digest such added substrata as gelatin or peptone, as in other gland structures.

5. The permanence of the protein structures of the brain appears to depend on the very perfect protection against asphyxia and  $\text{CO}_2$  accumulation, which its large blood supply and its control of respiration insures. When asphyxia and acidosis do develop in brain tissue, it autolyzes like other tissues.

6. The autolytic disintegration of the delicate protein structures of brain tissue appears to be an irreversible phenomenon, and is accompanied by loss of such characteristic functions as memory, habit, motor control, and consciousness.

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## A STUDY OF THE CATALASE REACTION.

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(Received for publication, May 2, 1921.)

The significance of catalase in the living organism is still an open question. Only one fact has thus far been incontestably established; namely, that catalase liberates molecular oxygen from hydrogen peroxide, though it fails to do so with similarly constituted organic compounds like ethyl hydrogen peroxide.

In a sense catalase lends itself admirably to the investigation of the dynamics of its action. The course of the reaction can be easily and directly timed and measured by the evolution of oxygen. Furthermore, the interference generally caused through an accumulation of products of the reaction is absent here since, except for very minute traces of oxygen remaining in solution, the gaseous by-product is quickly driven off, especially when the mixture is vigorously shaken.

### I.

All the experiments reported in this paper were performed with a crude catalase preparation from liver.<sup>1</sup> The finely ground pulp of beef liver was extracted with chloroform-water over night and the liquid was strained through cloth to remove solid particles. Enough alcohol was then added to this liquid to make a 50 per cent mixture, when a massy precipitate forms on standing. This precipitated material is collected on a hardened filter paper and the excess fluid removed by suction. The material is then dried in the air and ground to a fine powder in a mortar. The powder possesses strong catalase action. During several months the

<sup>1</sup> In some experiments with kidney extracts which were preliminary to this research the author enjoined valuable aid from Dr. V. E. Levine (*Science*, lii, 612).

reactivity of the preparation has apparently remained unchanged. A more highly purified sample of catalase can be prepared from this powder by reprecipitating a watery extract with an equal volume of alcohol. Since repeated precipitation causes great loss of the catalase, the crude preparation was used exclusively in these experiments. A definite quantity of this dry powder was weighed out (usually 0.5 gm.) and extracted with 200 cc. of chloroform-water. A water-clear extract is obtained which, when kept in an ice chest, retains its strength undiminished for a considerable time. The extract was neutralized with 0.01 *N* NaOH to make its pH = 7.0. The hydrogen ion concentration in all these experiments has been determined colorimetrically with the aid of the Hynson, Westcott and Dunning standards. The relative amount of catalase was measured by the number of cc. of the extract employed.

The hydrogen peroxide used in the experiments was the grade known under the trade name of "Oakland Dioxogen." Inasmuch as the hydrogen peroxide is strongly acid it was first neutralized with NaOH and its hydrogen ion concentration adjusted to a pH somewhat below 7.0. The reason for this procedure is that the hydrogen peroxide decomposes spontaneously when it is neutral or alkaline in reaction. On this account also just enough peroxide was prepared for the day's work; a fresh supply being made for each occasion. The strength of the hydrogen peroxide was standardized by titrating against 0.1 *N* potassium permanganate, and expressed in terms of its oxygen content. When a series of experiments lasted several hours the titer of the hydrogen peroxide was again checked up at the end of the experiment.

## II.

The apparatus in which the catalase activity was measured (Fig. 1) consisted of three parts: (1) a shaker driven by an electric motor; (2) an Erlenmeyer flask in which the reaction between the catalase and the hydrogen peroxide took place; and (3) a eudiometer tube in which the gas was collected for measurement. The volume of gas was reduced to standard conditions of temperature and pressure, and all the data recorded in the paper represent the corrected volume. The catalase preparation was

measured into the Erlenmeyer flask and enough water added to secure the desired relative concentration. In some experiments the same volume was maintained throughout the entire series. The hydrogen peroxide was measured into a glass cup provided with a syphon. The cup was suspended from the bottom of a rubber stopper with which the Erlenmeyer flask was closed. The stopper bore a plunger which could be easily pushed down into the cup by pressing on the rod extending to the outside. The descent of the plunger raised the level of the liquid in the cup and started the syphon which drained the contents in about 10 seconds. As soon as the cup was emptied the shaking was

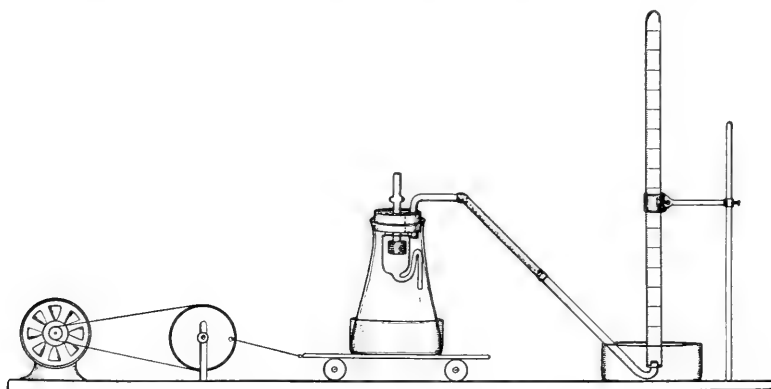


FIG. 1.

started by turning the motor switch. The evolution of the gas was recorded at the end of every 5 minutes, and the shaking continued until no more gas was given off. Usually experiments required about one-half to three-quarters of an hour for completion.

### III.

To insure its stability the commercial hydrogen peroxide is strongly acidified. When this acid hydrogen peroxide is used a serious error is introduced into the experiments, which seems to have been the case in the older experiments on catalase. Even when hydrogen peroxide is made neutral to Congo red, the peroxide is still strongly acid as far as its hydrogen ion concentration

is concerned. Certainly, in a series of experiments where the quantity of hydrogen peroxide is varied, the range of variation in hydrogen ion concentration may be very great. To avoid such a possibility, all our experiments were conducted at a definite hydrogen ion concentration. This end was accomplished either with the aid of appropriate phosphate buffer mixtures, or by bringing both the hydrogen peroxide and the catalase solution to a definite pH by adding 0.01 N NaOH. The objection to the use of the buffer is that, especially at a pH over 7.0, there is danger of the hydrogen peroxide being broken up by it. While within the short time of the catalase experiment this danger is greatly minimized, nevertheless, the second method was used in preference except where a wide range of pH values was required.

The first thing, of course, was to determine the optimum hydrogen ion concentration for the catalase reaction. Experiments were performed in which the only variable factor was the pH of the mixture, adjusted by means of phosphate buffers. The pH of the mixture was invariably checked up at the close of each experiment.

The series reported below has been made with 5 cc. of the catalase extract and hydrogen peroxide sufficient to yield 173 cc. of oxygen (determined by titration); 20 cc. of the appropriate buffer mixture were used in each experiment. The initial hydrogen peroxide concentration was 0.31 gram-molecular in all these experiments. For reasons which will become apparent from subsequent discussion the quantity of hydrogen peroxide and catalase was so adjusted that the curve of the oxygen evolution followed very closely the isotherm of a bimolecular reaction

$$K = \frac{1}{at} \cdot \frac{x}{a-x}$$

It is obvious from these experiments that the optimum condition for the catalase reaction is at neutrality (pH = 7.0). As the acidity of the medium increases, both the velocity of the reaction and the total amount of peroxide decomposed diminish somewhat at first, but when the pH is below 6.0 the decrease becomes very noticeable. Thus, at pH = 6.4 the reaction is 98 per cent complete, but at pH = 5.8 it is 88 per cent, and at pH = 5.2 it is only 70 per cent of that at the pH = 7.0. In-



creasing the hydroxyl concentration (pH = 7.2 to 8.3) has apparently little or no effect on the amount of hydrogen peroxide decomposed, though the reaction velocity is somewhat diminished.

TABLE I.

pH	Time.	Oxygen evolved.		$K \times 10^5$	$H_2O_2$ decomposed.
		Observed.	Calculated.		
	<i>min.</i>	<i>cc.</i>	<i>cc.</i>		<i>per cent</i>
5.2	5	50.7	44.5	48	55
	10	75.1	70.8	44	
	15	88.9	88.1	41	
	20	94.0	100.4	34	
	23	95.3	109.2	31	
				—	
				40	
5.8	5	62.5	56.5	66	69
	10	90.2	85.2	63	
	15	105.2	102.5	60	
	20	113.3	114.1	55	
	25	117.1	122.5	48	
	31	119.7	129.8	42	
				—	
				56	
6.4	5	62.9	63.5	66	76
	10	93.6	92.9	68	
	15	113.4	109.8	73	
	20	124.2	120.8	74	
	25	128.3	128.6	66	
	29	131.2	134.3	63	
	33	132.7	137.0	58	
				—	
				67	
7.0	5	65.1	65.8	70	79
	10	95.3	95.3	71	
	15	114.3	112.4	75	
	20	125.2	122.8	75	
	25	130.9	130.4	72	
	29	133.9	135.9	69	
	34	136.1	140.2	63	
				—	
				71	

TABLE I—*Concluded.*

pH	Time.	Oxygen evolved.		$K \times 10^3$	$\text{H}_2\text{O}_2$ decomposed.
		Observed.	Calculated.		
	<i>min.</i>	<i>cc.</i>	<i>cc.</i>	<i>per cent</i>	<i>per cent</i>
7.2	5	60.3	59.7	62	75
	10	89.9	88.8	63	
	15	108.2	106.1	64	
	20	119.4	117.4	64	
	25	126.2	125.4	62	
	31	130.0	131.2	56	
				—	
7.5	5	60.1	60.5	62	78
	10	88.2	89.6	60	
	15	108.0	106.9	64	
	20	120.6	118.2	67	
	25	128.3	126.3	62	
	30	132.8	132.2	64	
	35	135.2	136.9	59	
8.3	5	59.1	60.5	60	79
	10	86.4	89.6	58	
	15	107.8	106.9	65	
	20	120.0	118.2	65	
	25	127.6	126.3	65	
	30	133.0	132.2	64	
	35	136.1	136.9	60	
				—	
				62	

The fact of particular significance, however, is that while the reaction runs true to the course of a bimolecular curve within the range of hydrogen ion concentrations of from 6.4 to 8.3 (and about three-quarters of the peroxide undergoes decomposition), the reaction deviates from this course as the acidity exceeds  $\text{pH} = 6.4$ .

Although  $\text{pH} = 7$  has been found to be the optimum hydrogen ion concentration for the catalase reaction, the experiments were made in a medium slightly on the acid side ( $\text{pH} = 6.7$  to  $6.9$ )

in order to guard against spontaneous decomposition of the hydrogen peroxide which happens most readily at  $\text{pH} = 7.0$  and above.

#### IV.

An examination of the effect of changing the quantity or the concentration of the hydrogen peroxide on the catalase reaction brings to light a number of interesting points. When a series of experiments is performed with a constant quantity of catalase it becomes at once apparent that, as the hydrogen peroxide increases, the reaction proceeds more slowly. Furthermore, except where there is a considerable excess of catalase, only a certain and variable portion of the peroxide will be decomposed. Indeed, with every increase in the amount of hydrogen peroxide there is a diminution in the proportion which is decomposed with the liberation of its oxygen. This will be seen from a series of experiments with a constant amount of catalase and a variable amount of hydrogen peroxide, recorded graphically in Fig. 2.

These experiments were made with 5 cc. of the catalase preparation. The total volume of the mixture was 50 cc., and the reaction of the mixture was maintained at  $\text{pH} = 6.9$  throughout the series. The temperature in this as well as all other experiments was  $20\text{--}21^\circ\text{C}$ . In this series the hydrogen peroxide used represented a range of variation of from 45 to 226 cc. of available oxygen, as determined by titration with permanganate. Since the final volume was the same in all experiments, the concentration of the peroxide varied from 0.08 to 0.40 gram-molecular. The smallest concentration was so much below the catalase capacity that it has been left out of the graphic record. With a gram-molecular concentration of 0.16 (*i.e.*, with 90 cc. of oxygen available) 94.2 per cent of the hydrogen peroxide was decomposed in 12 minutes when the reaction came to an end. With an increase in the concentration up to 0.32 gram-molecular the amount of oxygen evolved gradually rises but the proportion of hydrogen peroxide decomposed diminishes. Thus, while the amount of oxygen liberated increases from 85 to 122.7 cc. the percentage of hydrogen peroxide decomposed diminishes from 94.2 to 68.2 per cent. When the concentration of the hydrogen peroxide is greater than 0.32 gram-molecular, the quantity of

oxygen actually set free in the reaction begins to decline. At concentrations below 0.16 gram-molecular the catalase is so much

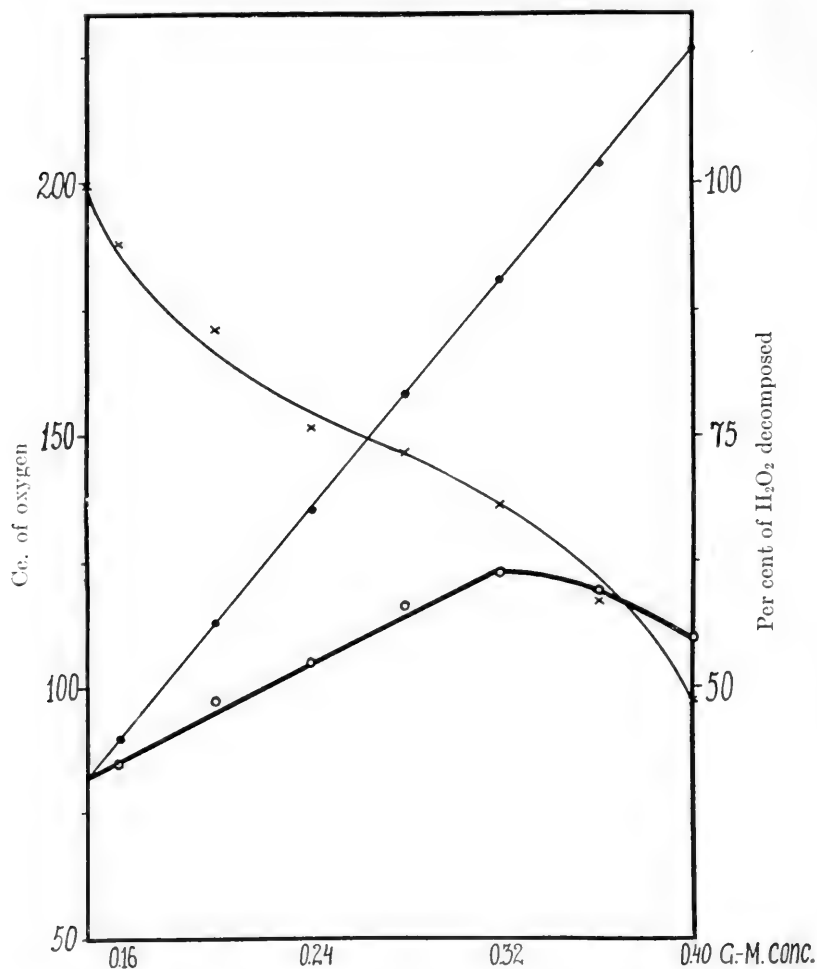


FIG. 2. Effect of increasing quantities of hydrogen peroxide on the catalase reaction. •—• Cc. of oxygen available. ○—○ Cc. of oxygen liberated. ×—× Per cent of hydrogen peroxide decomposed.

in excess that the hydrogen peroxide is completely decomposed. Considering the regularity of the curve, it is reasonable to assume

that with hydrogen peroxide equivalent to 85 cc. of oxygen the decomposition would be 100 per cent complete.

The decline in the absolute quantity of oxygen liberated with the same amount of catalase requires further consideration. This may have been either due to an increase in the amount of hydrogen peroxide or to its greater concentration. It might be supposed, for instance, that the peroxide contains some impurity which is too negligible when the quantity of peroxide is small, but when more of this is used there is also enough of the injurious substance to retard and depress the reaction. Such a view, however, is untenable because it can be shown that the depressing effect may be produced by the same quantity of hydrogen peroxide when its concentration is increased. Thus in an experiment with the same amounts of peroxide and of catalase the volume was so adjusted that in one case the peroxide was in a concentration of 0.18 and in the other of 0.36 gram-molecular. In the former case the reaction ended with the production of 87.3 cc. of oxygen, while in the latter, only 78.2 cc. were set free. On the other hand, it must be emphasized that the change in concentration of the peroxide is not the only and effective factor in depressing the reaction. Even with a constant concentration of peroxide, as soon as the hydrogen peroxide quantity has been increased beyond a certain point, the reaction is progressively depressed.

#### V.

The depressing influence exercised by hydrogen peroxide on the catalase reaction has been invariably observed also by the older investigators of the subject. It has been generally noted that the amount of oxygen evolved diminishes, but the effect was attributed by them to a destruction of the catalase through oxidation by the excess of hydrogen peroxide. Indeed, the fear of oxidizing the catalase with the hydrogen peroxide was so predominant in the earlier work on catalase that the reaction was carried out at very low temperatures (0°C.) and with very dilute hydrogen peroxide solutions. This has occasioned certain errors in interpretation to which we shall return later. In the older experiments on catalase no heed was paid to the hydrogen ion concentration of the reaction medium. Since peroxide is

usually strongly acid, and the relative acidity would increase with the increase in the quantity of the hydrogen peroxide employed, it is not improbable that the very great depression observed under these conditions was primarily the effect of greater acidity. The considerable depression (a loss of 30 per cent in catalase activity) brought on by a change in the reaction of the medium only from pH 7 to 5.2 has already been demonstrated in the foregoing.

Experiments in which the depressing effect was produced by merely increasing the concentration of the peroxide without actually changing its quantity convinced us that the apprehension that the catalase may be oxidized by the peroxide is entirely unfounded. But to test this matter further a series of experiments was performed in which instead of a small excess of hy-

TABLE II.

Hydrogen peroxide.		Oxygen evolved in minutes.				H <sub>2</sub> O <sub>2</sub> decom- posed.
Concen- tration.	Oxygen.	10	20	30	40	
<i>mol</i>	<i>cc.</i>	<i>cc.</i>	<i>cc.</i>	<i>cc.</i>	<i>cc.</i>	<i>per cent</i>
0.2	112	53.5	75.8	85.8	91.2	82.1
0.4	224	49.6	60.7	74.9	76.6	34.2
0.8	448	30.3	43.7	53.2	57.5	13.6
1.6	896	23.0	29.9	30.3	34.1	3.8
2.4	1,344	19.5	23.0	23.7		1.8

drogen peroxide *very large* excesses were used. These experiments prove definitely the inadequacy of the idea of the destruction of the catalase through oxidation with hydrogen peroxide. In the experiments reported below hydrogen peroxide of such strength was used that it bleached and even blistered the skin of the hands. One could naturally expect that a few milligrams of catalase would be quickly and completely destroyed by so powerful an oxidizing reagent. This, however, was not the case, and the reaction, though much retarded and suppressed, continued for a considerable length of time, as can be seen from the data in Table II.

It is obvious from these results, therefore, that the diminished catalase activity which occurs when the hydrogen peroxide is increased beyond a certain optimum quantity can be accounted

for neither on the assumption that it is due to some incidental impurity, nor that the catalase undergoes destructive oxidation. Two things, however, can be regarded as proven: First, that when the quantity of hydrogen peroxide is above a certain minimum, which the catalase can break up completely, the amount of oxygen evolved gradually rises to a maximum point with an increase in the amount of hydrogen peroxide. In a number of experiments it has been established that with our liver catalase preparation the maximum oxygen evolution occurs when 65 to 70 per cent of the total hydrogen peroxide is decomposed. Secondly, when the maximum evolution of oxygen has been reached, further increases in the quantity of the hydrogen peroxide, even when not considerable, cause a falling off in the amount of oxygen set free in the reaction. This fact permits apparently of only one interpretation; namely, that we are dealing with a reversible reaction. This view will receive further support when we consider the nature of the catalase reaction.

#### VI.

The question naturally presented itself whether or not all the catalase is used up in the reaction when 65 to 70 per cent of the hydrogen peroxide breaks up setting free its oxygen. The following experiments were made with this in view. 5 cc. of the liver catalase solution were allowed to react with a quantity of hydrogen peroxide equivalent to 173 cc. of oxygen. In 30 minutes the reaction was complete when 120.1 cc. of oxygen were set free. In other words, 69 per cent of the hydrogen peroxide was decomposed. If free catalase were still present in the mixture it would be impossible to explain why the remaining 52.9 cc. failed to be liberated. Indeed, when another 5 cc. of catalase are now added to the system from which no more oxygen came off, the previously undecomposed quantity breaks up very rapidly. In 8 minutes 52.1 cc. of oxygen are given off which is practically the theoretically expected amount. We must, therefore, conclude that when the reaction came to a stop with the evolution of 120.1 cc. of oxygen that there was no more active catalase present in the system. The experiment, under precisely the same conditions, was then repeated with a certain modification. If

a certain amount of catalase can decompose only a definite quantity of hydrogen peroxide and is thereby itself used up, will this happen also when the reaction occurs in more than a single stage?

5 cc. of the catalase preparation were again allowed to react but this time with only about half the quantity of hydrogen peroxide (92 cc. of  $O_2$ ). The reaction went off much quicker than before, and in 10 minutes it was all over while practically the entire theoretical amount of oxygen was set free (91.7 cc.). More hydrogen peroxide was then added to bring the total amount of available oxygen to 180 cc. of oxygen. Upon the addition of the second quantity of hydrogen peroxide the reaction started up once more, this time, however, much more slowly, and in 15 minutes 26.4 cc. were liberated. Altogether, therefore, 118.1 cc. of oxygen were given off in the two consecutive stages, which compares very favorably with the former result, when the entire peroxide quantity reacted at once with the catalase. The somewhat smaller amount of gas formed may perhaps be due to the fact that in the second experiment there was about 5 per cent more peroxide than before. The experiment was then tried with still another variation. Starting with a quantity of hydrogen peroxide equivalent to 180 cc. of oxygen it was made to react with only 3 cc. of the catalase preparation. The reaction ran to completion in 29 minutes with the liberation of 71.2 cc. of oxygen. There was still, therefore, an excess of 108.8 cc. present. When now 2 more cc. of the catalase were added to the mixture, the reaction commenced again but at a very slow rate and ceased in 25 minutes when 52.3 cc. of oxygen were liberated. Thus, 123.5 cc. of oxygen were produced, or 69 per cent of the peroxide decomposed, when 5 cc. of catalase were used though the reaction occurred now in two stages. The conclusion seems, therefore, justifiable that the catalase is used up in the reaction, and that it reacts with a definite quantity of hydrogen peroxide. The reaction is essentially the same as that between an acid and a base, or perhaps more correctly as between an alcohol and a fatty acid. It likewise seems highly probable from our evidence that when the hydrogen peroxide is 65 to 70 per cent decomposed the liver catalase is completely used up.



## VII.

The study of the relation between varying amounts of catalase and the quantity of oxygen liberated from a definite amount of hydrogen peroxide gains particular interest in the light of the

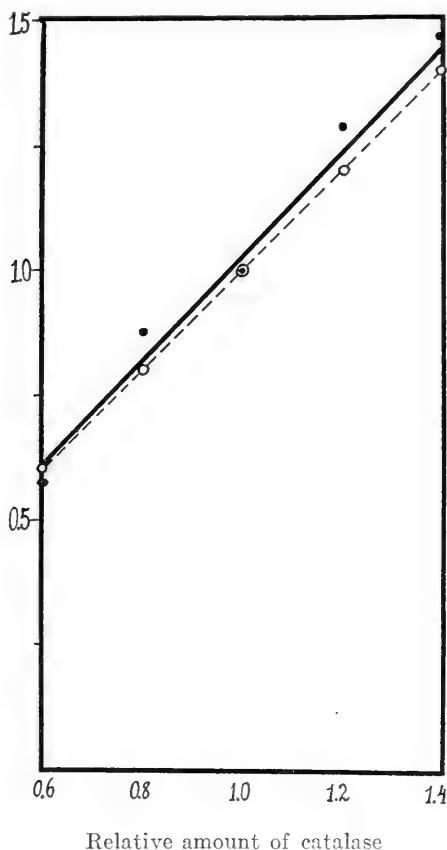


FIG. 3. •• Relative amount of oxygen set free. ○—○ Relative concentration of catalase.

experiments just discussed. In the preceding series of experiments using a constant catalase quantity and different amounts of hydrogen peroxide it was found that, at any rate within a limited range of variations of the peroxide, there is a regular

## Catalase Reaction

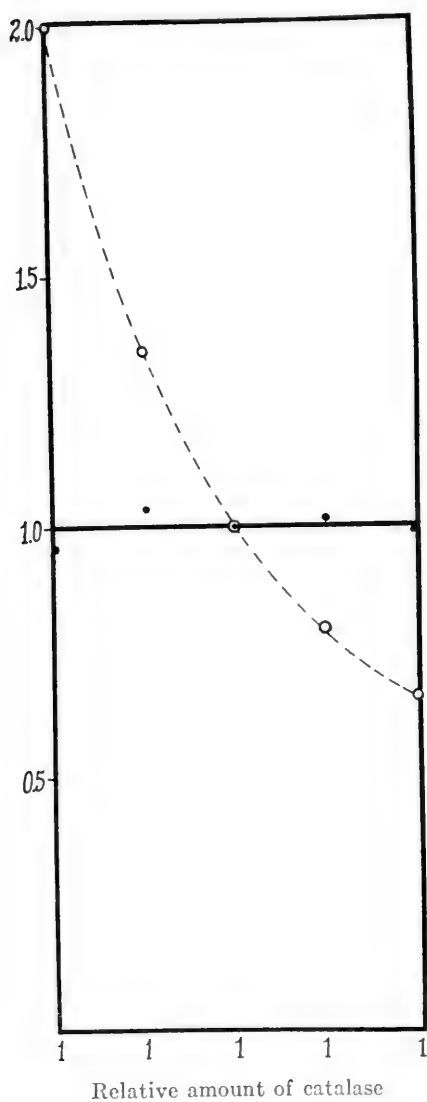


FIG. 4. —•— Relative amount of oxygen set free. —○— Relative concentration of catalase.

increase in the oxygen set free. When, however, a constant quantity of peroxide is employed it is found that the degree of its decomposition depends upon the absolute quantity of catalase and that the quantity of oxygen liberated is directly proportional to the catalase. The direct relationship between the varying amounts of catalase and the oxygen given off from a definite

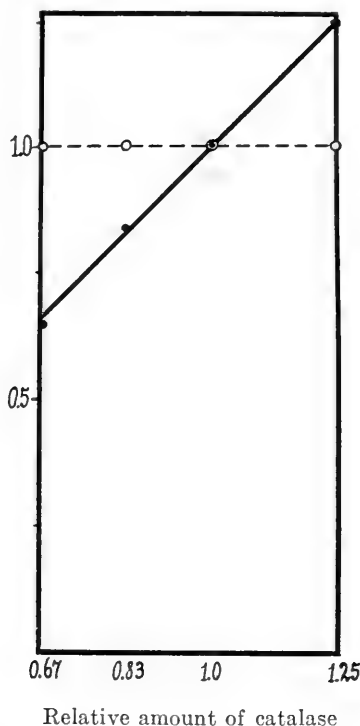


FIG. 5. ●—● Relative amount of oxygen set free. ○—○ Relative concentration of catalase.

quantity of hydrogen peroxide is demonstrated by a number of experiments which are graphically recorded in Figs. 3, 4, and 5. These experiments represent a variety of conditions, the quantity and concentration of the catalase varying either separately or simultaneously, while the concentration of the peroxide also is either changed or kept constant. The curves are plotted with the relative quantities of catalase as abscissæ and the corres-

ponding relative quantities of oxygen set free at the time the reaction has been completed as ordinates. The relative concentration of the catalase is also indicated in each graph.

It is clear from these experiments that there is a direct proportionality between the amount of catalase and the amount of oxygen it produces from a given quantity of hydrogen peroxide. This proportionality holds only for the absolute quantity of catalase,<sup>2</sup> changes in concentration alone having no direct effect on the oxygen set free. This furnishes, therefore, additional evidence for the view that the catalase reacts with a definite quantity of hydrogen peroxide.

#### VIII.

The amount of hydrogen peroxide decomposed (or the amount of oxygen liberated) is a linear function of the quantity of catalase provided a constant amount of hydrogen peroxide is used in the experiments (its concentration need not be constant). With the same quantity of catalase, however, changing the quantity of hydrogen peroxide (or even changing its concentration beyond a certain point) will limit the catalase reaction to a greater or less extent. This, of course, is true only in the case when the hydrogen peroxide is already present in such excess that it is less than 65 per cent decomposed. Within the limits of complete decomposition (100 per cent) and two-thirds decomposition (65 to 70 per cent) the amount of oxygen set free from the hydrogen peroxide increases but not in direct proportion to the increase of its quantity. This can be seen from the data recorded in the first part of Table III as well as from the graph in Fig. 6. On the other hand, when the decomposition of the hydrogen peroxide used falls below 65 per cent the amount of oxygen produced by the same quantity of catalase diminishes with every increase in the hydrogen peroxide. The data in the second part of Table III demonstrate this point. Plotting the results with the relative quantity of hydrogen peroxide as the abscissæ and the

<sup>2</sup> This rule holds true only when the quantity of catalase is neither too small nor too large for the amount of hydrogen peroxide employed. In these events the disturbing factors already discussed dominate the reaction and there is no longer a direct proportionality between the catalase and the quantity of oxygen liberated from the hydrogen peroxide.

relative amount of oxygen formed by the catalase as well as the per cent of decomposition of the peroxide as ordinates, we find that the curve of the oxygen evolution has a smaller slope for every new increase in the amount of hydrogen peroxide employed.

The greatest diminution in the evolution of oxygen from hydrogen peroxide by a certain quantity of catalase occurs, therefore, with the relatively smaller excess of peroxide. This fact is significant because if the diminution were due to an oxidation of the catalase a proportionately greater destruction of it could be expected with the greater amount of hydrogen peroxide.

TABLE III.

Hydrogen peroxide.		Oxygen evolved from $H_2O_2$ .	
Oxygen available.	Relative amount.	Relative amount.	Absolute amount.
<i>cc.</i>			<i>cc.</i>
90	1.00	1.00	85
113	1.26	1.14	97
135	1.50	1.22	104.5
158	1.76	1.37	116
181	2.00	1.43	122.7
112	1.00	1.00	92.0
168	1.50	0.90	82.1
224	2.00	0.83	76.6
448	4.00	0.66	60.8
896	8.00	0.37	34.1
1,344	12.00	0.26	23.7

## IX.

The rate of the catalase reaction can be determined by comparing the lengths of time required to liberate a definite quantity of oxygen under different conditions (see Osterhout). The results of a number of experiments were plotted and the time necessary to produce 25, 50, 75, or 100 cc. of oxygen was measured from the graphs. It is understood, of course, that the reaction velocity is the reciprocal of the time. In Table IV the results are given of a number of tests performed under varied experimental conditions. This study extended to the effect upon the reaction rate of constant and varying quantities of catalase (with or with-

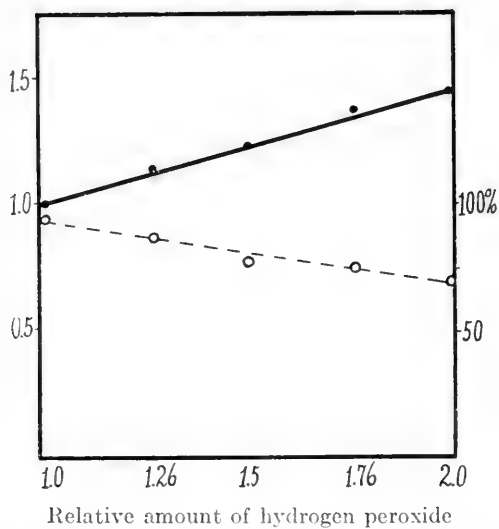


FIG. 6. ●—● Relative amount of oxygen set free. ○—○ Per cent of hydrogen peroxide decomposed.

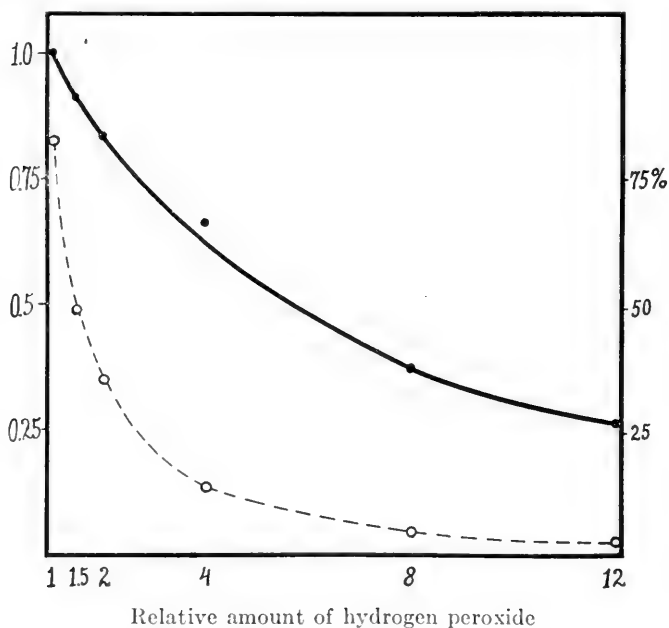


FIG. 7. ●—● Relative amount of oxygen set free. ○—○ Per cent of hydrogen peroxide decomposed.

out change of concentration), and of constant and variable quantities of hydrogen peroxide (with or without changing its concentration).

TABLE IV.

Catalase.		Hydrogen peroxide.		Time required to evolve oxygen.			
Quantity.	Relative concentration.	Concentration.	Oxygen available.	25 cc.	50 cc.	75 cc.	100 cc.
cc.		mol	cc.	min.	min.	min.	min.
5	1.0	0.16	90	1.20	3.60	8.5	—
5	1.0	0.20	113	1.55	4.00	8.0	—
5	1.0	0.24	136	1.70	4.25	8.6	18.1
5	1.0	0.28	158	1.85	4.75	8.9	15.8
5	1.0	0.32	181	1.80	4.50	8.8	15.8
5	1.0	0.36	203	1.85	4.60	8.7	15.4
5	1.0	0.40	226	2.35	6.00	11.7	21.7
9	0.67	0.12	100	3.2	9.3	—	—
9	0.80	0.14	100	2.9	?	27.0	—
9	1.00	0.18	100	2.9	8.5	28.0	—
9	1.33	0.24	100	2.9	8.9	27.8	—
9	2.00	0.36	100	3.2	9.5	—	—
6.75	1.0	0.13	112	1.60	4.10	8.6	—
5.63	1.0	0.16	112	1.90	5.20	11.9	—
4.50	1.0	0.20	112	2.75	8.20	19.2	—
3.75	1.0	0.25	112	3.50	11.40	—	—
3.00	1.0	0.30	112	5.50	25.00	—	—
3	0.6	0.20	108	2.5	7.2	21.2	—
4	0.8	0.26	145	2.0	5.0	10.7	22.4
5	1.0	0.32	180	1.5	4.0	8.5	15.0
6	1.2	0.39	217	1.3	3.2	5.8	9.5
7	1.4	0.42	254	1.2	2.9	5.5	9.0
3	0.6	0.32	181	4.25	10.75	—	—
4	0.8	0.32	181	1.90	4.85	10.1	21.6
5	1.0	0.32	181	1.50	4.10	8.6	15.2
6	1.2	0.32	181	0.75	2.30	4.5	7.6
7	1.4	0.32	181	0.45	1.60	—	5.5

Increasing the quantity of hydrogen peroxide (also its concentration), while the catalase remains the same, causes a certain amount of retardation of the reaction particularly during the

early stages. Thus, in the first experiment the hydrogen peroxide quantity increased from an equivalent of 90 cc. to that of 203 cc. of available oxygen, while the hydrogen peroxide was thus more than doubled, the reaction velocity diminished somewhat, and only 1 minute more was required to liberate 50 cc. of oxygen (4.6 instead of 3.6 minutes). At a later stage in the reaction this difference disappears, and the time required to set free 75 cc. of oxygen is practically the same (about 8.5 minutes). However, when the concentration of the hydrogen peroxide reaches 0.4 gram-molecular the slowing up of the reaction becomes very pronounced and persists through the entire reaction.

When the experiment is performed with varying relative concentrations of catalase (maintaining a constant quantity) while keeping the same quantity of hydrogen peroxide (second experiment), we find that the rate of the reaction remains practically unaltered. To decide whether the constancy of the reaction velocity is due to the fact that the quantity of the hydrogen peroxide or that of the catalase is the same, an experiment was performed in which the relative concentration of the catalase was maintained unchanged through the series (third experiment) while the absolute quantities varied from 3.0 to 6.75 cc. of the extract. The quantity of hydrogen peroxide in the meantime was kept constant. Under these circumstances the rate of the reaction (reciprocal of the time) was found to vary directly with the catalase quantity. The concentration of the catalase is evidently of no particular consequence so far as the reaction velocity is concerned. Two other experiments were made with varying quantities of catalase while both the concentration and quantity of the hydrogen peroxide either varied or were kept constant. Plotting the velocities against the quantities (also the concentrations) of the catalase (Figs. 8 and 9), these results corroborate further the findings of the previous experiments demonstrating definitely that the reaction rate depends directly on the quantity of catalase used, while the effect of the hydrogen peroxide is to limit the rate. This conclusion seems, therefore, a corollary to that derived from the study of the rôle played respectively by the catalase and the hydrogen peroxide in the reaction.



## X.

The catalase reaction is generally regarded as belonging to the monomolecular order, only one substance—hydrogen peroxide—undergoing decomposition. Though it is true that the reaction may follow the monomolecular curve, the widely accepted idea that from a dynamic standpoint the catalase reaction is of this

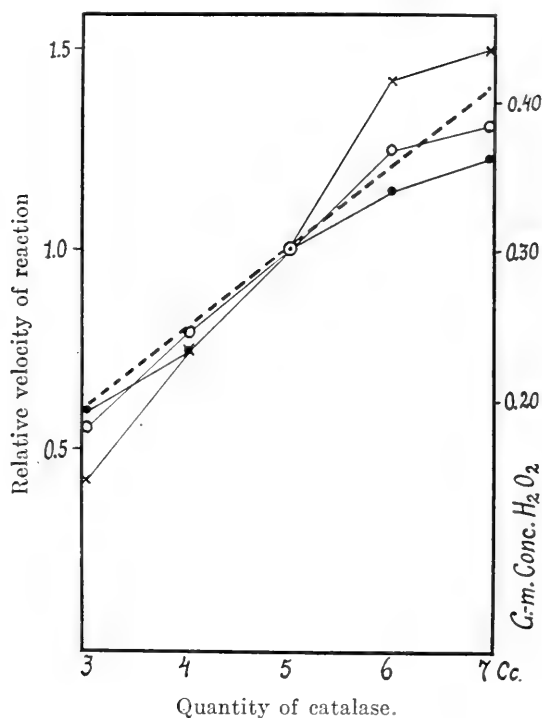


FIG. 8. Effect of changes in catalase on reaction velocity. •—• 25 cc. of oxygen set free. ○—○ 50 cc. of oxygen set free. ×—× 75 cc. of oxygen set free.

particular type requires drastic revision. The evidence presented in this paper goes to show that not only does the hydrogen peroxide disappear but that the catalase as well is used up in the course of the reaction, and that a definite quantitative relation exists between the interacting catalase and hydrogen peroxide. Furthermore, when the time relations of the evolution of oxygen,

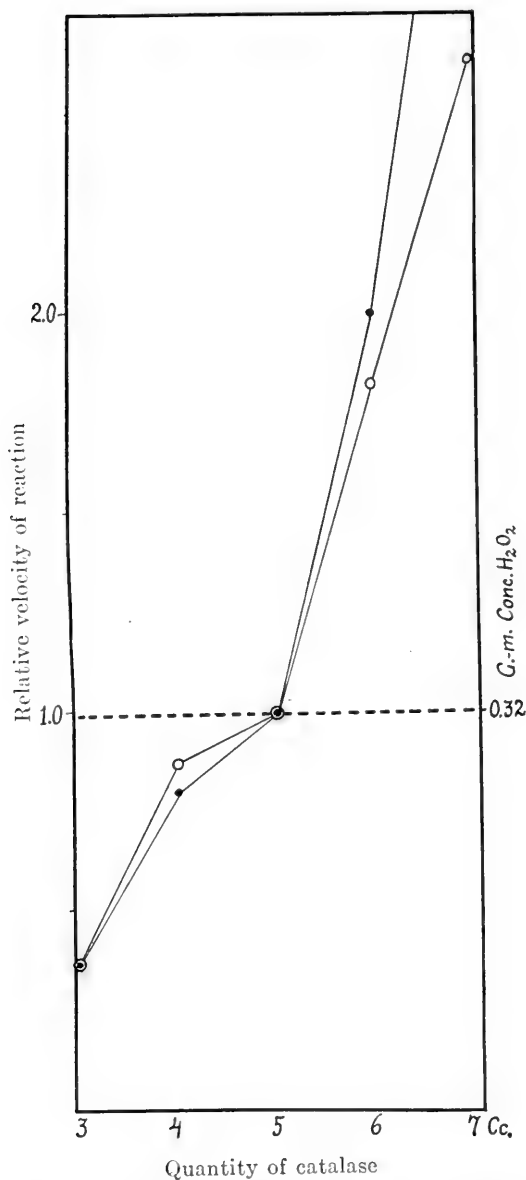


FIG. 9. Effect of changes in catalase on reaction velocity. The quantity and concentration of hydrogen peroxide is unchanged. ●—● 25 cc. of oxygen set free. ○—○ 50 cc. of oxygen set free.

resulting from the reaction of catalase with hydrogen peroxide, are studied under different experimental conditions, and the reaction is followed not for a limited space of time but until the reaction stops, it is soon discovered that it does not invariably follow the monomolecular isotherm. As will be shown presently, the reaction under certain conditions is bimolecular and even one and a half molecular, while under other conditions it does not conform to either of these types. In fact, the course of the reaction seems to depend solely on the quantitative relation between the available catalase and the hydrogen peroxide. Where the former is in excess, the hydrogen peroxide will be completely decomposed; on the contrary, when the hydrogen peroxide is in excess, a greater or less proportion of it will undergo decomposition. Barring from the present discussion either extreme, *i.e.* cases where there is too great an excess of catalase or of hydrogen peroxide, for which I was unable to find a simple mathematical expression of the reaction, we will confine our attention only to such instances where the decomposition of the hydrogen peroxide ranges from about 65 to 100 per cent of the quantity used. Within this limited range the reaction does not run true to any one particular dynamic formula. When 95 to 100 per cent of the oxygen available in the hydrogen peroxide is liberated, the reaction is unquestionably monomolecular, and the reaction coefficients can be determined from the well known formula,  $K = \frac{1}{t} \cdot \log \frac{a}{a-x}$ . When the catalase is present in large excess, and this can usually be detected immediately by the extreme vigor and velocity of the reaction, this no longer applies, while there also occur other changes which will be discussed separately in a forthcoming paper. The fact, however, has been definitely established that in the presence of a small excess of catalase the reaction runs true to the monomolecular course. Herein we find the reason why the older observers were led to believe that the reaction is of this particular order. Guided by the erroneous notion that catalase may be destroyed through oxidation by hydrogen peroxide, they experimented with very dilute peroxide solutions which were completely decomposed by the catalase. In other words, the experiments were confined chiefly to a particular range of catalase-peroxide relationship, and this fact was

TABLE V.  
Catalase 5 cc. Total volume 50 cc.

Hydrogen peroxide.		Time.	Oxygen evolved.	Reaction coefficient.*	H <sub>2</sub> O <sub>2</sub> decomposed.
Oxygen available.	Concentration.				
cc.	mol	min.	cc.	$K_1 \times 10^4$	per cent
90	0.16	5	64.4	1092	95
		10	81.6	1046	
		12	85.0	1030	
				1056	
113	0.20			$K_2 \times 10^4$	86
		5	63.1	95	
		10	85.6	97	
		15	95.1	94	
		18	97.0	87	
				93	
135	0.24			$K_3 \times 10^5$	76
		5	58.5	107	
		10	82.5	113	
		15	96.5	121	
		20	103.3	116	
		24	104.5	112	
158	0.28	5	56.7	67	73
		10	81.6	66	
		15	99.5	71	
		20	110.4	72	
		25	115.2	67	
		27	115.9	69	
181	0.32	5	56.5	50	68
		10	81.6	46	
		15	99.5	46	
		20	110.6	44	
		25	118.0	42	
		30	122.0	39	
		34	122.7	44.7	

\*The designations  $K_1$ ,  $K_2$ , and  $K_3$  are employed as abbreviations for the coefficients of unimolecular, one and a half molecular, or bimolecular reactions, respectively.

TABLE V—*Concluded.*

Hydrogen peroxide.		Time.	Oxygen evolved.	Reaction coefficient.	H <sub>2</sub> O <sub>2</sub> decomposed.
Oxygen available.	Concentration.				
cc.	mol	min.	cc.		per cent
203	0.36	5	56.0		58
		10	82.9		
		15	100.5		
		20	111.0		
		25	117.1		
		30	119.0		
226	0.40	5	47.7		49
		10	69.4		
		15	86.6		
		20	97.8		
		25	105.2		
		30	109.1		
		33	109.7		

responsible for the unqualified assumption that the catalase reaction is of this particular type.

When, however, the catalase-hydrogen peroxide ratio is so adjusted that less than 95 per cent of the available oxygen is set free, the reaction is no longer monomolecular. Either by diminishing the quantity of catalase or by increasing the quantity of hydrogen peroxide any degree of decomposition may be secured, as was already expounded in the foregoing. When the decomposition is somewhere between 85 and 95 per cent of the hydrogen peroxide (the limits are not sharply defined) the course of the reaction follows the curve of a one and a half molecular, and the reaction coefficient can be determined from the formula,

$$K = \frac{1}{t} \cdot \frac{\sqrt{a} - \sqrt{a-x}}{\sqrt{a(a-x)}}.$$
 When the decomposition is somewhere between 70 and 80 per cent of the hydrogen peroxide used in the experiment the reaction runs true to the bimolecular course, and  $K = \frac{1}{t} \cdot \frac{x}{a(a-x)}$  applies within these limits. Since it has already been shown that when the decomposition of the hydrogen peroxide falls below 65 per cent the actual amount of oxygen set free by the catalase as well as the reaction velocity are considerably

diminished, it is obvious that we are dealing not simply with a bimolecular reaction but also one that is apparently reversible.<sup>3</sup>

Before discussing this matter further a few experiments will be recorded in which either a constant quantity of catalase was used with varying amounts of hydrogen peroxide or a constant quantity of hydrogen peroxide while the catalase was varied.

<sup>3</sup> From theoretical considerations Yamazaki also comes to the conclusion that depending upon the relative amounts of hydrogen peroxide and catalase the type of the reaction may shift from the bimolecular to the monomolecular. In his calculations he nevertheless employs the equation for a monomolecular reaction. This he does even where, as will be shown, the equation does not apply. Recalculating his data given in Table 76 (IV and V) where with 10 and 5 cc. of the catalase preparation, respectively, he obtained 90 and 75 per cent of decomposition of the hydrogen peroxide, it can be shown that the reaction follows the equation of either a one and a half or of a bimolecular reaction as was also found to be the case with my preparation of liver catalase. I may add that since the paper had been sent to press I had occasion to experiment with catalase preparations from different sources and was able to substantiate the results in every instance. In the tabulation below the values of the constant ( $K$ ) as found by Yamazaki with the aid of the monomolecular formula and those which I calculated in accordance with the bi-, or one and a half molecular equation are set down side by side. The point is brought out so clearly that no further comment is required.

	Time.	$A$	$X$	$K$ (mono- molec- ular).	$K$ (bimolec- ular).
	<i>min.</i>				
5 cc. catalase, 75 per cent de- composition.	0	12.52			
	5.08	12.52	2.20	0.0159	0.00335
	9.58	12.52	3.58	0.0145	0.00334
	15.16	12.52	4.80	0.0114	0.00328
	20.83	12.52	5.87	0.0114	0.00339
	26.08	12.52	6.54	0.0088	0.00335
					$K$ (One and a half molec- ular).
10 cc. catalase, 90 per cent de- composition.	0	13.33			
	4.03	13.33	3.24	0.0357	0.0102
	13.11	13.33	8.72	0.0276	0.0113
	18.46	13.33	9.18	0.0245	0.0119
	23.86	13.33	10.11	0.0204	0.0118

The results under both kinds of conditions are the same. The experiments were all made under a uniform temperature (20–21°C.) and similar hydrogen ion concentration of the medium (pH = 6.7 to 6.9).

TABLE VI.  
Catalase 4.5 cc.

Hydrogen peroxide.		Time.	Oxygen evolved.	Reaction coefficient.	H <sub>2</sub> O <sub>2</sub> decomposed.
Oxygen available.	Concentration.				
cc.	mol	min.	cc.	$K_1 \times 10^4$	per cent
87	0.2	5	36.5	472	100
		10	54.3	425	
		15	66.0	412	
		20	74.2	416	
		30	82.3	423	
		40	86.2	509	
		45	87.1	443	
112	0.2			$K_3 \times 10^5$	82
		5	36.7	88	
		10	53.5	82	
		15	67.0	89	
		20	75.8	93	
		30	85.8	97	
		40	91.2	98	
168	0.2	45	92.0	91	49
		5	36.4		
		10	53.4		
		20	74.5		
		30	80.7		
224	0.2	35	82.1		34
		10	49.6		
		20	67.6		
		30	74.9		
		35	76.6		

A review of these data shows that the results may be calculated in accordance with different dynamic formulas depending upon the degree of decomposition of the hydrogen peroxide effected in the reaction. The results of the four series of experiments are presented diagrammatically in Fig. 10.

TABLE VII.

Hydrogen peroxide equivalent to 112 cc. O<sub>2</sub>.

Catalase.	Time.	Oxygen evolved.	Reaction coefficient.	H <sub>2</sub> O <sub>2</sub> decomposed.
<i>cc.</i>	<i>min.</i>	<i>cc.</i>	$K_1 \times 10^4$	<i>per cent</i>
6.75	5	56.6	620	100
	10	80.1	545	
	15	94.1	531	
	20	102.1	559	
	25	106.3	517	
	30	108.8	515	
	40	111.8	548	
			$K_2 \times 10^4$	
5.63	5	49.0	63	93
	10	69.3	59	
	15	82.1	59	
	20	91.5	63	
	25	97.5	67	
	30	101.0	69	
	35	103.7	61.7	
			$K_3 \times 10^5$	
4.5	5	37.4	90	79
	10	55.1	88	
	15	67.4	90	
	20	76.1	95	
	25	80.7	92	
	30	83.7	88	
	35	85.8	84	
	45	88.0	90	
3.75	10	47.1		63
	20	63.5		
	30	69.2		
	40	70.0		
3.0	10	33.8		51
	20	46.4		
	30	53.2		
	40	56.4		
	45	57.2		



We can recognize (Fig. 10) three distinct zones in the diagram corresponding to 68 to 82 per cent decomposition, 88 to

TABLE VIII.

Hydrogen peroxide equivalent to 181 cc. of  $O_2$ .

Catalase.	Time.	Oxygen evolved.	Reaction coefficient.	$H_2O_2$ decomposed.
cc.	min.	cc.	$K_1 \times 10^4$	per cent
7	5	88.7	573	100
	10	131.0	560	
	15	155.2	567	
	20	169.1	596	
	25	176.7	662	
	30	180.7	592	
6			$K_2 \times 10^4$	88
	5	78.2	49	
	10	112.2	46	
	15	135.0	49	
	20	147.0	49	
	25	153.4	47	
5	30	157.0	44	68
	34	158.3	47.3	
			$K_3 \times 10^5$	
	5	56.5	50	
	10	81.6	46	
	15	99.5	46	
4	20	110.6	44	59
	25	118.0	42	
	30	122.0	39	
	33	122.7	44.7	
	10	74.8		
	20	98.0		
3	30	105.5		39
	33	106.3		
3	10	47.6		39
	20	66.6		
	30	71.2		

93, and 95 to 100 per cent. A different formula applies for each zone, and for this zone alone. We may consider, therefore, that the catalase reaction is bimolecular changing to monomo-

lecular, the middle zone merely representing the transition from the one to the other. Mellor recites a number of instances where the reaction shows a similar change. He says<sup>4</sup> that "the gradual approach of the velocity curves for the bimolecular reaction to the curves for a unimolecular reaction as the amount of one of the reacting components of the bimolecular reaction is increased, shows very clearly how the course of a bimolecular reaction might appear unimolecular when one of the reacting components is in excess." Among reactions of this kind Mellor mentions the

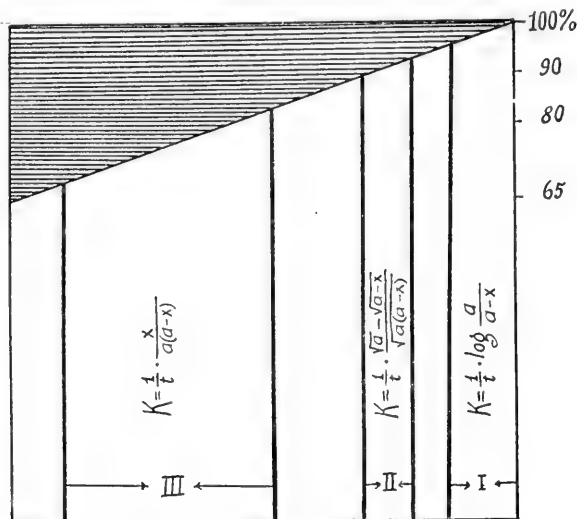


FIG. 10. Diagram showing the order of reaction depending on the degree of decomposition of hydrogen peroxide. The shaded area represents the undecomposed fraction of hydrogen peroxide.

reduction of potassium permanganate with an excess of oxalic acid; the action of an excess of hydrogen peroxide on hydriodic acid; etc. The catalase reaction seems to belong to the same category except that in this instance it is not the excess of hydrogen peroxide but of the catalase which shifts the reaction from the order of a bimolecular to that of a unimolecular.

Evans in a very thorough study of the catalase reaction (which he regards simply as a catalytic decomposition of hydrogen perox-

<sup>4</sup> Mellor, p. 42.

ide) observed that the reaction coefficients, calculated according to the formula  $\frac{dt}{dx} = K(a-x)$ , showed a peculiar behavior. In some instances the values of  $K$  would form an ascending, sometimes a descending series, and only occasionally did he obtain fairly constant values. He distinguished, therefore, three periods: the rectilinear, the infralogarithmic, and the logarithmic, a terminology which does not seem happily chosen. The latter is the one when the reaction really follows the curve of a monomolecular reaction. Examining his data, I find that this condition was rarely met, and only when the substrate ( $H_2O_2$ ) was used in very dilute solution while the amount of enzyme was "not very small." In other words, when there was enough catalase to completely decompose the hydrogen peroxide. The three phases which Evans noted are all related to the catalase-peroxide ratio. I found that the same three phases occur no matter whether the reaction follows the monomolecular, bimolecular, or an intermediate course. Whenever the catalase is present in too great an excess the values of  $K$  (monomolecular) will continually increase. On the other hand, when there is less catalase than is necessary to decompose the entire amount of peroxide the values of  $K$  gradually diminish. This is likewise true when the values of  $K$  are calculated for a one and a half or for a bimolecular reaction. The change of phase is brought about very readily by the smallest alteration in quantity of either the catalase or the hydrogen peroxide. With a little experience it is possible to adjust the quantities so as to make the reaction proceed according to any of the three formulas employed in this paper for calculating the reaction coefficient. It is remarkable how little it is necessary to change the quantities to produce appreciable differences in the values of  $K$ . Sometimes a change by a few drops of the catalase preparation was quite sufficient to bring this result about. When one bears in mind that with a decomposition of the hydrogen peroxide of about 75 to 80 per cent the reaction follows almost ideally the bimolecular curve, it is a simple matter indeed to adjust conditions so that the results of an entire experimental series can be made really comparable. In the study of the effect of the concentration of hydrogen ions upon the reaction it was already pointed out that the respective

quantities of both the catalase and the hydrogen peroxide were so chosen that the reaction was typically bimolecular. It is by virtue of this adjustment that it was possible to demonstrate in that series that increasing the hydrogen ion concentration not only limits the decomposition of hydrogen peroxide and slows up the reaction, but that the type of the reaction also changed and it no longer followed the course of a bimolecular curve when the pH fell below 6.0.

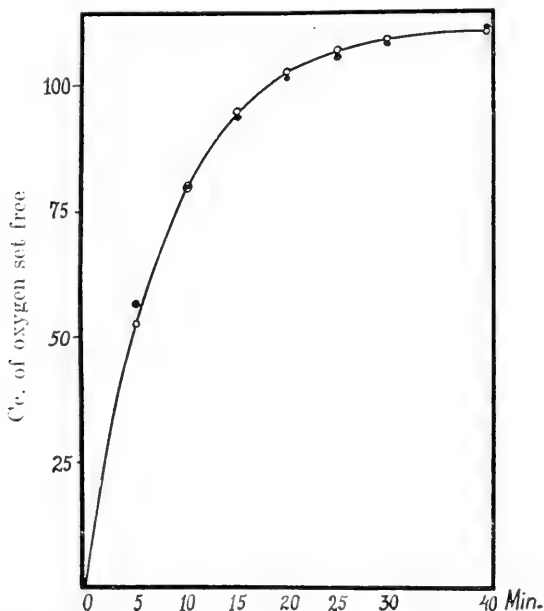


FIG. 11. Table VII, Experiment 1. •—• Experimental results. ○—○ Calculated results.

In Figs. 11, 12, and 13, the experimental curves are plotted together with the theoretical curve calculated with the aid of the average value of  $K$  for each respective experiment. The similarity of the two curves requires no further comment.

Before closing the paper it may be well to point out the bearing of the results upon the question of the technique of catalase determinations. The use of very large quantities of hydrogen peroxide (equivalent to 500 or 600 cc. oxygen), as is practised

commonly in researches on catalase, is objectionable because the depressing effect is great unless very large amounts of the catalase preparation are employed. There is no obvious advantage in working with such tremendous quantities. On the other hand, when an attempt is made to compare the catalase activity of preparations of presumably different strengths, the depressing effect will be much greater in the case of the weaker sample and under such conditions the tendency will be to exaggerate the

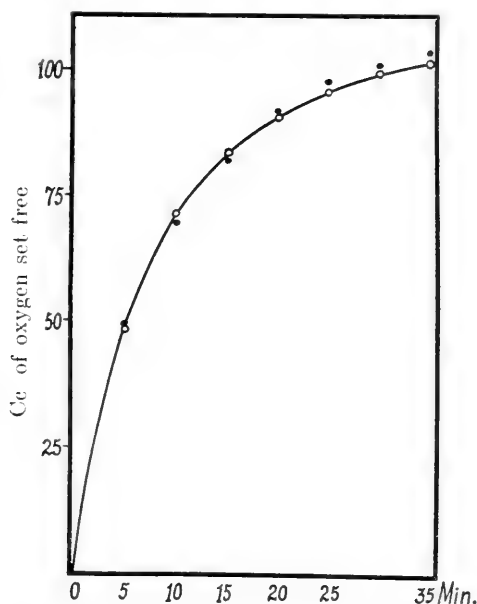


FIG. 12. Table VII, Experiment 2. •—• Experimental results. ○—○ Calculated results.

differences. In fact the entire method of comparing several samples of catalase on the basis of the amount of oxygen which they respectively liberate from hydrogen peroxide is of questionable accuracy. The comparison should instead be made between respective quantities of catalase preparation required to set free the same amount of oxygen from a given quantity of hydrogen peroxide. It is further advisable to adjust the reaction to follow some definite course (a 75 per cent decomposition of the hydrogen peroxide is a very good basis). Although the oxygen formation

is a linear function of the quantity of catalase, this rule does not hold true when either the catalase or the hydrogen peroxide are in great excess. When, however, the catalase is varied to produce a certain degree of decomposition with the same quantity of peroxide, the catalase strengths will be inversely proportional to the quantities used for the tests. The method of estimating the catalase strength followed in most investigations on catalase is so crude and untenable from a chemical standpoint that one naturally is reluctant to accept the conclusions drawn from

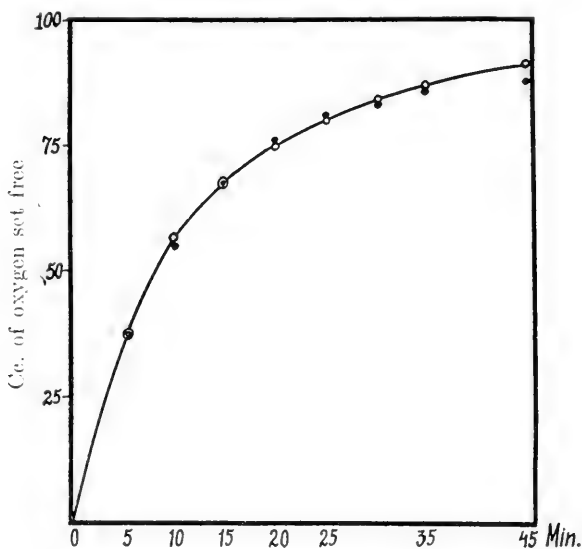


FIG. 13. Table VII, Experiment 3. •—• Experimental results. ○—○ Calculated results.

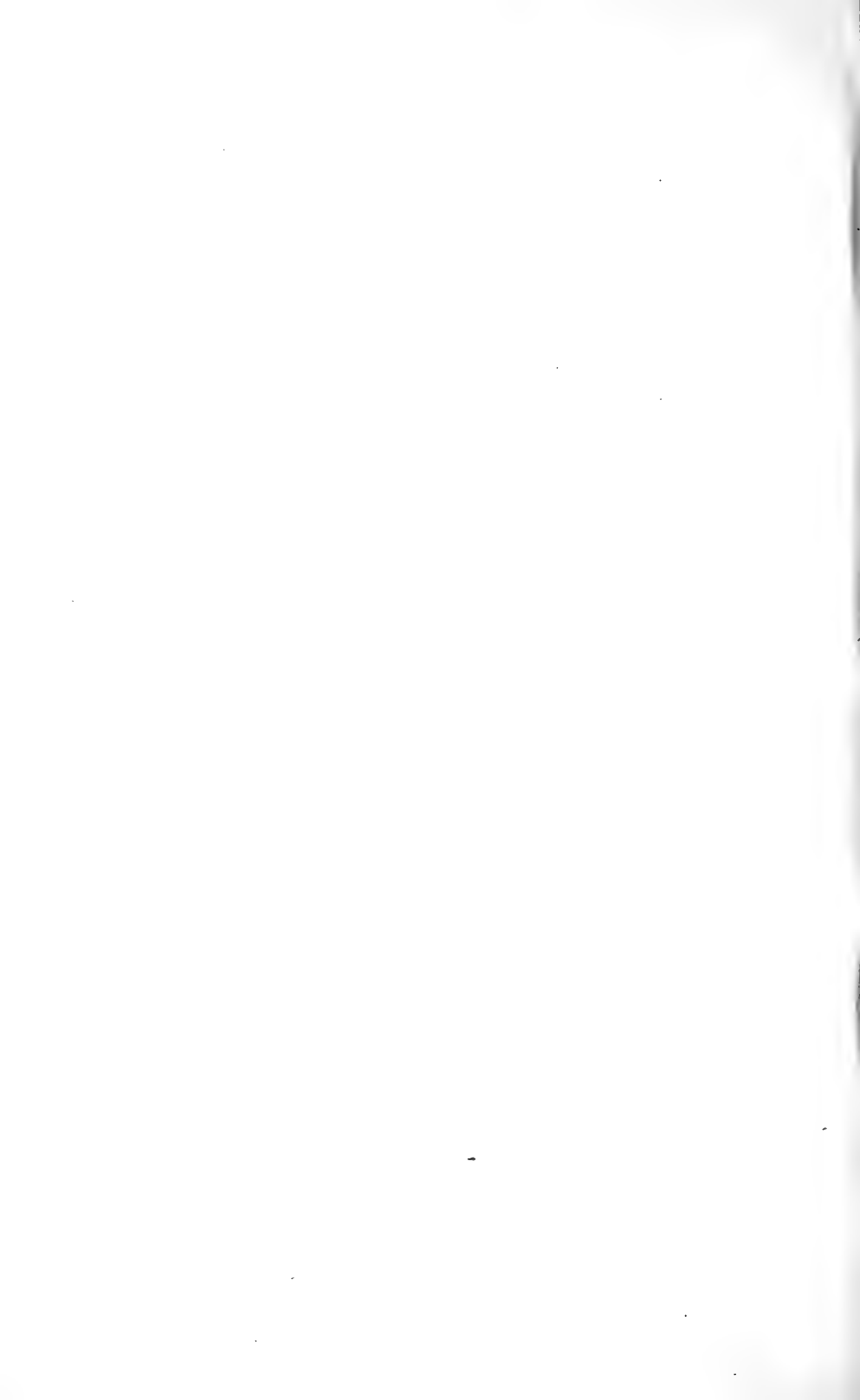
those researches, especially where the conclusions are of far reaching significance.

Another important matter is the fact that in the catalase experiments the reaction is allowed to go on for a set length of time, usually 10 or 15 minutes. The reasons for determining relative reaction speeds from the time periods required to effect a given result, rather than from the results effected in a given time period have been fully discussed by Osterhout, and need not be repeated. It should be noted, however, that when the

reaction is permitted to run for 10 minutes, only 50 or perhaps 60 per cent of the reaction is completed. For comparative purposes, it may be argued, this will not matter, and this might be true if the reaction were always brought to the same end-point. This is not likely to be the case. If, on the other hand, the reaction is so vigorous and rapid as to be completed within the 10 or 15 minutes it is practically certain that there is a great excess of catalase. In this event, of course, the oxygen evolution will again fail to give a correct measure of the relative catalase strengths inasmuch as there is no means of determining whether or not the relative excess is the same in each instance. Little credence can therefore be given to results of catalase experiments unless very large differences are demonstrated.

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# THE RELATION OF THE MIGRATION OF IONS BETWEEN CELLS AND PLASMA TO THE TRANSPORT OF CARBON DIOXIDE.\*

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(Received for publication, May 23, 1921.)

Due to the interest attached to the relationship of the chloride-bicarbonate equilibrium (Henderson, 1921) to the transport of carbon dioxide by the blood, we are publishing some of our data on this subject. Van Slyke and Cullen (1917) showed that the plasma of whole blood which had been shaken with varying proportions of carbon dioxide had lost an amount of chloride which was sufficient to account for about two-thirds of its increase in bicarbonate. More recently, Fridericia (1920) has published data which show that the loss of chloride more nearly accounts for the gain in bicarbonate. Henderson and coworkers (1920) found that only 60 per cent of the increase of bicarbonate was due to the migration of chlorine.

A study of this subject and permeability of cells in general were uppermost in mind when one of us started the adaptation of methods of inorganic analysis 3 years ago.<sup>1</sup> At that time the existing methods of determining sodium and potassium were laborious and required excessive amounts of material. We are now able to make a study of the permeability of cells to the more important anions and cations on small samples of blood.

\* The data in this paper are taken from a thesis presented by Miss Eaton in partial fulfillment of the requirements for the degree of Master of Science, Washington University, 1921.

<sup>1</sup> A study of methods of analysis of inorganic substances of biochemical importance was begun in the fall of 1918 by R. D. Bell and the senior author of this paper. Procedures for the determination of sodium and phosphorus have been published. Complete details of the systematic inorganic analyses of blood will soon be submitted to the Journal for publication.

Practically all the investigators in this field have admitted the permeability of red cells to hydrochloric acid when the blood is treated with carbon dioxide. According to Hamburger (1916) and coworkers this effect is extended to include sulfates, phosphates, sodium, potassium, and calcium. A number of investigators (Gürber, 1895) have failed to find an exchange of cations between cells and plasma. Lately, Van Slyke (1921) and Henderson and coworkers (1920) have stated that the migration of cations probably plays no part in the transport of carbon dioxide. While we are not in a position to state emphatically that there is *no transfer*, our analytical data show clearly that quantitatively the shift, if it occurs at all, is *very small*.

### *Methods.*

For our investigation of this problem we have devised a scheme of inorganic analysis which permits the determination of sodium, potassium, chlorine, and phosphorus on 10 cc. of serum. An additional quantity of 3 cc. is necessary for bicarbonate determinations.

Briefly the method is: Transfer 10 cc. of serum to a 100 cc. flask containing about 70 cc. of distilled water. Add while shaking 10 cc. of 20 per cent trichloroacetic acid.<sup>2</sup> Make to mark and mix well. After 30 minutes filter through dry paper. Since the filtration is rather slow, it is essential that both the funnel and flask be covered to avoid evaporation. Usually we obtain about 83 cc. of filtrate of which the following amounts are used for the determinations named below.

As may be seen from Table I we have utilized only 50 cc. of our filtrate; the remainder may be used for a repetition of doubtful analyses. We expect to extend this scheme to the determination of calcium and magnesium.

<sup>2</sup> We had already worked out independently the use of trichloroacetic acid for chloride analysis when Smith's (1921) paper appeared. Our results on these filtrates agree very closely with values obtained on picric acid filtrates from blood plasma. Invariably, however, the latter procedure gives results, which are 2 or 3 per cent higher on whole blood. We have not found time to study this intensively and are still a little undecided which method is the more nearly correct.

*Treatment of the Blood.*—In order to avoid as far as possible the effect of change in strength (as an acid) of the hemoglobin in passing from the oxidized to reduced form, we have generally maintained in our saturators a sufficient amount of oxygen to keep essentially all of the hemoglobin as oxyhemoglobin. When the higher tensions of carbon dioxide were used, oxygen was added from a tank to maintain its concentration between 20 and 21 per cent. Barcroft (1914) has shown that about 13 per cent of oxygen is sufficient to keep over 90 per cent of the hemoglobin in the oxidized form in the presence of 11 per cent of carbon dioxide. We think that 20 per cent oxygen was sufficient to prevent the reduction of more than 10 per cent of the oxyhemoglobin in all of our experiments in which the gas mixture contained not more than 20 per cent carbon dioxide.

TABLE I.

Analysis.	Volume of filtrate used.	Method.
	cc.	
Sodium.....	10	Sodium cesium bismuth nitrite, Doisy-Bell (1921).
Potassium.....	20	Cobalti-nitrite.
Chlorine.....	10	McLean-Van Slyke solutions (1915).
Phosphorus.....	10	Colorimetric, Bell-Doisy (1920).
Total.....	50	

Beef blood was defibrinated and filtered through several layers of gauze, and 60 cc. samples were measured out into a series of  $2\frac{1}{2}$  liter bottles. At least 6 liters of the gas mixtures were passed through each bottle and the openings closed with pinch-cocks. The inlet tube extended nearly to the bottom of the bottle, the outlet being flush with the stopper. As our chief aim was to study the shift of inorganic ions from serum to corpuscles under higher tensions of  $\text{CO}_2$ , we did not analyze the gas mixtures in the saturators in any except the last experiment. These analyses revealed the fact that 6 liters were insufficient to bring the gas to even the approximate per cent of  $\text{CO}_2$  given in our tables. Our 20 per cent  $\text{CO}_2$  mixture probably sufficed to make the gas in the saturator not over 15 per cent  $\text{CO}_2$ . The bottles were

rotated to secure a maximum surface for the rapid attainment of equilibrium. After agitation for about 20 minutes the blood was drawn off under oil into centrifuge tubes.

For the determination of corpuscle volume, we used graduated tubes of 6 mm. bore. All samples of the blood were centrifuged simultaneously for 30 minutes at 3,500 R.P.M. immediately after equilibration. Our centrifuge was occasionally tested to show that this speed was actually attained. The volume of corpuscles was read and the tubes were again centrifuged for 15 minutes. If comparison showed that any appreciable decrease had occurred the tubes were centrifuged again. The serum was immediately removed for analysis.

The various analyses were started and the bicarbonate determinations finished on the same day that the blood was taken. One of us (E. P. E.) determined the chlorides; the other (E. A. D.), the bicarbonates.

In general, the bicarbonate determinations were made according to the technique described by Van Slyke and coworkers (1919), using a standard pH 7.2 and phenolsulfonephthalein. It seemed preferable to titrate to a standard pH than to use the various gas mixtures required for this purpose if the Van Slyke-Cullen (1917) method were used. This point is mainly theoretical in nature, but will assume some importance in the larger changes of bicarbonate concentration.

We are giving one of our experiments in its entirety at this point to aid in the discussion of our results. Other similar data are given in the protocols to strengthen our conclusions.

*Corpuscle Volume.*—By reference to Table II it can be seen that the volume of corpuscles increased with increasing tensions of  $\text{CO}_2$ . This effect which was emphasized by Hamburger (1916) has not been confirmed by Joffe and Poulton (1920). The reason for their exceedingly variable results is not clear to us. In none of our experiments have we failed to find an increase in corpuscle volume with increasing tensions of  $\text{CO}_2$ . This is probably due to the fact that the osmotic pressure of the cell contents is increasing more rapidly than that of the serum. The serum does not show a net gain of ions; for each molecule of  $\text{NaHCO}_3$  gained a molecule of  $\text{HCl}$  is lost. The corpuscles, however, gain this molecule of  $\text{HCl}$ .

TABLE II.

*Experiment 6.*—Defibrinated beef blood equilibrated with various air-carbon dioxide mixtures at 25°C. Centrifuged for 45 minutes.

No hemolysis. Bicarbonate by titration. Figures in column headed, CO<sub>2</sub>, signify the amount of carbon dioxide in the 6 liters of gas passed through the saturators.

Treatment of whole blood.	Corpuscles.	NaCl		NaHCO <sub>3</sub> concentration.	Na per 100 cc.	K per 100 cc.	P per 100 cc.
		Per 100 cc.	Concentration.				
CO <sub>2</sub> per cent	per cent	mg.	mol	mol	mg.	mg.	mg.
3	42.9	623	0.1068	0.0293	322	20.9	7.9
10	43.1	610	0.1043	0.0317	319	21.3	7.8
20	43.5	602	0.1030	0.0345	326	21.3	7.8
100	45.5	564	0.0965	0.0431	345	22.9	7.8

Correction for change in volume of corpuscles.

$$10 \text{ per cent } \frac{571}{569} \times 0.0293 = 0.0294 \text{ M NaHCO}_3 \text{ expected.}$$

$$\times 0.1068 = 0.1071 \text{ M NaCl} \quad "$$

$$20 \text{ per cent } \frac{571}{565} \times 0.0293 = 0.0296 \text{ M NaHCO}_3 \quad "$$

$$\times 0.1068 = 0.1079 \text{ M NaCl} \quad "$$

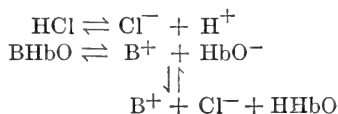
$$100 \text{ per cent } \frac{571}{545} \times 0.0293 = 0.0307 \text{ M NaHCO}_3 \quad "$$

$$\times 0.1068 = 0.1119 \text{ M NaCl} \quad "$$

Effect of increasing CO<sub>2</sub> content of air from:

	3 to 10 per cent		3 to 20 per cent		3 to 100 per cent	
	BCl	BHCO <sub>3</sub>	BCl	BHCO <sub>3</sub>	BCl	BHCO <sub>3</sub>
	mol	mol	mol	mol	mol	mol
Before increase.	0.1071	0.0317	0.1079	0.0345	0.1119	0.0431
After "	0.1043	0.0294	0.1030	0.0296	0.0965	0.0307
	-0.0028	+0.0023	-0.0049	+0.0049	-0.0154	+0.0124

This molecule of hydrochloric acid probably reacts with the alkali oxyhemoglobinate in much the same way that the carbonic acid does.



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Instead of two we now have three units which exert osmotic pressure. This means a real gain and quite naturally water passes from the serum into the cells to equalize the osmotic pressure on both sides of the membrane.

When the hydrogen ion concentration passes the isoelectric point of oxyhemoglobin we have a very similar phenomenon. There is no net gain of ions in the serum but the increase continues in the cell due to the hydrochloric acid gained. This acid probably reacts with a molecule of hemoglobin which is now functioning as a base. Whereas, previously we had only an undissociated hemoglobin molecule, we now have two ions.



These schematic equations are intended to express only the increase in osmotic pressure of the cells. We do not intend that they shall convey our idea of the linkages between acids or alkalies and proteins. Other factors such as alterations of the degree of association of hemoglobin molecules may influence the changes of osmotic pressure.

It seems evident to us that the cause of swelling of the corpuscles is the increase of osmotic pressure within the cell. We should then expect water to pass in until both the serum and cell contents have the same osmotic pressure. The volume of serum per 100 cc. of blood is now less than formerly. All its constituents, provided no migration has occurred, should be present in greater concentration. According to this view, it is essential that corrections be introduced in our calculations for the change in volume of the corpuscles.

Reference to Table II shows that at 20 per cent  $\text{CO}_2$ , the corpuscle volume has increased 0.6 cc. per 100 cc. of whole blood. This amounts to slightly more than 1 per cent increase in the concentration of the serum salts. The serum volume at 3 per cent  $\text{CO}_2$  is 57.1 cc.; at 20 per cent  $\text{CO}_2$  it is 56.5 cc. Consequently the concentration of sodium, potassium, bicarbonate, chlorine, and phosphorus at 3 per cent  $\text{CO}_2$  must be multiplied by  $\frac{571}{565}$  to give the expected concentration at 20 per cent.

$$\frac{571}{565} \times 0.0293 = 0.0296 \text{ M NaHCO}_3 \text{ expected value.}$$

$$\frac{571}{565} \times 0.1068 = 0.1079 \text{ M NaCl} \quad \quad \quad \text{“} \quad \text{“}$$

$$\text{Gain in base by loss of HCl, } 0.1079 - 0.1030 = 0.0049 \text{ M}$$

$$\text{“} \quad \text{“} \quad \text{“} \text{ as NaHCO}_3, \quad 0.0345 - 0.0296 = 0.0049 \text{ M}$$

We have purposely taken an experiment where the agreement is better than usual. If we have failed to correct for the volume change of corpuscles the loss in chloride would account for only 73 per cent of the gain in bicarbonate.

$$\text{Gain in base by loss of HCl, } 0.1068 - 0.1030 = 0.0038 \text{ M}$$

$$\text{“} \quad \text{“} \quad \text{“} \text{ as NaHCO}_3, \quad 0.0345 - 0.0293 = 0.0052 \text{ M}$$

The foregoing calculations demonstrate the error caused by failure to correct for the loss of water from the serum. The discrepancy, of course, will be much larger in experiments where the corpuscle volume change is larger; *i.e.*, at 100 per cent CO<sub>2</sub>.

It is evident that mathematically the accurate determination of corpuscle volume is of great importance if one wishes to study the true relationship between the direct and indirect methods of determination of increased base in plasma. For instance, if the volume of corpuscles found had been 44.1 per cent instead of the 43.5 per cent then our calculations would have been:

$$\frac{571}{559} \times 0.0293 = 0.0299 \text{ M NaHCO}_3 \text{ expected value.}$$

$$\frac{571}{559} \times 0.1068 = 0.1091 \text{ M NaCl} \quad \quad \quad \text{“} \quad \text{“}$$

$$\text{Gain in base by loss of HCl, } 0.1091 - 0.1030 = 0.0061 \text{ M}$$

$$\text{“} \quad \text{“} \quad \text{“} \text{ as NaHCO}_3, \quad 0.0345 - 0.0299 = 0.0046 \text{ M}$$

From a very close agreement between the values, an error of about 1 per cent in determination of plasma volume produces results which are rather widely divergent.

The foregoing series of calculations illustrates why the previously published figures have shown that the shift of hydrochloric acid freed an amount of base equivalent to only about two-thirds or three-fourths of the increase of bicarbonate. When we started our work we fully expected to find the remainder accounted for by a passage outward of sodium from the corpuscles.

However, we now feel that such an explanation is entirely superfluous.

These results make it appear more than probable that in as far as the plasma functions as a carrier of carbon dioxide (Joffe and Poulton, 1920; Smith, Means, and Woodwell, 1921; and Fridericia, 1920) the transport is based entirely (providing the  $[H^+]$  of the plasma remains constant) upon the passage of hydrochloric acid back and forth across the cell membrane. That this mechanism is possible is closely related to the preeminent buffer value of hemoglobin due to its change of dissociation constant in passing from oxyhemoglobin to reduced hemoglobin.

#### *Permeability of Blood Corpuscles.*

We have found it quite easy to repeat the demonstration of the permeability of cells to the chloride ion. Naturally one would expect this effect to be extended to include other anions. However, when one considers the concentration of the other inorganic anions in serum ( $P=0.002$  M;  $S=0.002$  M) it is evident how little importance they would probably have in the transport of carbon dioxide.

Though we have not attempted to decide upon the permeability to the sulfate ion, we have made a few colorimetric determinations of inorganic phosphate. Although nearly all of our results point to a migration of phosphate they are not decisive in that most of the differences are within the possible experimental error. Our one gravimetric determination indicates that no shift of inorganic phosphate occurs. We are very loth to draw any conclusion from this but will extend our work in this direction.

With respect to a shift of cations, Hamburger (1916) concluded from the results of a few experiments that carbon dioxide causes a passage of potassium into the cells and of sodium outward into the plasma. Previous workers (Gürber, 1895) had failed to detect any shift of cations but Hamburger attributed this to a lack of suitable methods of analysis.

We fully expected to detect a transfer of both sodium and potassium. Quantitatively, Hamburger found that shaking horse blood with 20 volumes per cent of  $CO_2$  (20 cc. of pure  $CO_2$  to 80 cc. of blood) caused about 17 per cent of the potassium to



pass into the corpuscles and a 6 per cent increase of the sodium of the serum. While our experiments were not conducted exactly as Hamburger's were, we have a few data on blood treated in a comparable manner. We have shaken beef blood with 40 or 50 volumes of gas, containing proportions of  $\text{CO}_2$  varying from 5 to 100 per cent. In practically no case has any shift of either potassium or sodium been found.

We consider that our determinations of sodium have more significance than those of potassium. In case our duplicate results varied by more than 2 per cent other analyses were made. In general the values reported are the mean of two results which varied by less than 2 per cent. Such being the case a variation of 2 per cent of the value found from that expected may be considered a real shift. In some of our later experiments a deviation of 1 per cent may be taken as an exchange of sodium. By reference to the protocols it can be seen that only very infrequently does a shift of sodium seem to occur. In view of its rare occurrence and the difficulty of obtaining filter paper, etc., free from sodium we are inclined to ascribe this to experimental error.

Our determinations of potassium are less reliable than those of sodium. However, it is possible that any variation greater than from 2 to 3 per cent from the expected value is a real transfer of potassium. This rarely occurs.

We feel that our results lead to the conclusion that blood cells are permeable to the chloride ion but impermeable to both sodium and potassium. Collip (1921) has recently published data which also point in this direction.

#### DISCUSSION.

According to Van Slyke's (1921) recent review, hemoglobin seems to be by far the most important factor in the transport of carbon dioxide from the tissues to the lungs. As it is non-diffusible an auxiliary reaction is utilized to assist the plasma to take up or give off carbon dioxide. This mechanism is a shifting back and forth across the cell membrane of hydrochloric acid. As we picture the process it is dependent upon a very slightly varying hydrogen ion concentration of the plasma.

In the capillaries where the tension of carbon dioxide is high the hydrogen ion concentration of the plasma *tends* to increase.

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This produces the shift of HCl to the cells. At the same time there is the reduction of the oxyhemoglobin which alone would

TABLE III.  
*Chloride Bicarbonate Equilibrium.*

Experiment No.	Treatment of whole blood.	Plasma chloride.		Plasma bicarbonate.		Increase of base determined by.	
		Concentration found.	Concentration expected.	Concentration found.	Concentration expected.	Concentration loss in chloride.	Concentration gain in NaHCO <sub>3</sub> .
	CO <sub>2</sub> per cent	mol	mol	mol	mol	mol	mol
1	5	0.0332		0.0072			
	10	0.0327	0.0336	0.0081	0.0073	0.0009	0.0008
	20	0.0316	0.0344	0.0098	0.0075	0.0028	0.0023
2	5	0.0287		0.0112			
	20	0.0280	0.0292	0.0126	0.0114	0.0012	0.0012
3	3	0.0366		0.0097			
	15	0.0340	0.0370	0.0131	0.0098	0.0030	0.0033
4	3	0.1112		0.0274			
	10	0.1081	0.1136	0.0311	0.0280	0.0055	0.0031
	20	0.1063	0.1141	0.0352	0.0281	0.0078	0.0071
5	3	0.1090		0.0294			
	10	0.1071	0.1098	0.0318	0.0296	0.0027	0.0022
	20	0.1043	0.1107	0.0366	0.0298	0.0064	0.0068
	100*	0.0977	0.1139	0.0444	0.0307	0.0162	0.0137
6	3	0.1068		0.0293			
	10	0.1043	0.1071	0.0317	0.0294	0.0028	0.0023
	20	0.1030	0.1079	0.0345	0.0296	0.0049	0.0049
	100*	0.0965	0.1119	0.0431	0.0307	0.0154	0.0124
7	3	0.1076		0.0270			
	14	0.1020	0.1098	0.0351	0.0276	0.0078	0.0075
	21	0.0998	0.1111	0.0380	0.0279	0.0113	0.0101
	37*	0.0976	0.1131	0.0408	0.0284	0.0155	0.0124
	80*	0.0960	0.1135	0.0431	0.0285	0.0175	0.0146

\* Oxygen was considerably less than 20 per cent.

produce a more alkaline reaction within the cells. The two effects are normally so well balanced that there is practically no alteration in the [H<sup>+</sup>] of the blood.

In the lungs the reverse process occurs; namely, a loss of carbon dioxide from the plasma, oxygenation of the hemoglobin, and a shift of hydrochloric acid back to the plasma.

Our experiments were undertaken with the hope of being able to clear up the transfer of anions and cations back and forth across the cell membrane. In Table III we have grouped together our experimental data on the loss of chloride and gain of bicarbonate in the serum. Over what might be called a very extreme physiological range (3 to 15 per cent  $\text{CO}_2$ ) we find that the loss of serum chloride adequately accounts for the gain in bicarbonate in experiments conducted at room temperature ( $25^\circ\text{C}.$ ) When much higher percentages of carbon dioxide (50 to 100 per cent) were used this equivalence was not found. The loss of chloride invariably exceeded the gain in bicarbonate. We have no explanation to offer for this circumstance. The important feature to us is the equivalence over physiological ranges of carbon dioxide tensions.

In the one experiment in which the gas mixtures were analyzed our data agree with those of Hasselbalch and Warburg (1918) rather than with those of Haggard and Henderson (1920). We find that the shift of chloride with the accompanying increase of bicarbonate continues beyond 280 mm. of  $\text{CO}_2$ . Variable factors of temperature and species should be mentioned. These changes do occur to a greater extent for a given increase of  $\text{CO}_2$  at the lower than at the higher tensions.

In Table IV we have placed our results on the shift of phosphate, sodium, and potassium. With the possible exception of phosphate, our data indicate an impermeability of the cell membrane to these ions under our experimental conditions.

We do not mean to give the impression that the corpuscles are impermeable under all circumstances. Such a condition seems to be rather improbable. However, we do think that migration of sodium and potassium plays no part in the transport of carbon dioxide. The permeability of cells to both phosphates and sulfates has not been settled. Our data with respect to the former are not conclusive.

In view of some recent comments on the ash of plasma (Melanby and Thomas, 1920) and the possibility of sodium proteinates we are grouping some of our data in Table V to show the excess of cations over anions.

## 388 Ion Migration between Cells and Plasma

TABLE IV.

*Effect of Various Carbon Dioxide Tensions on the Migration of Sodium, Potassium, and Phosphorus.*

Experiment No.	Treatment of whole blood.	Na per 100 cc.		K per 100 cc.		P per 100 cc.	
		Found.	Expected.	Found.	Expected.	Found.	Expected.
	<i>CO<sub>2</sub> per cent</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>
2	5	249				78.9	
	20	253	253			80.5	80.3
3	3	150					
	15	151	152				
4	3	336		20.7		7.4	
	10	336	340			7.4	7.5
	20	350	345	21.6	21.2	7.3	7.6
5	3	327		21.1		8.3	
	10	331	329	21.1	21.2	8.3	8.3
	20	332	332	21.1	21.4	8.3	8.4
	100*	345	341	23.8	22.0	8.3	8.7
6	3	322		20.9		7.9	
	10	319	323	21.3	21.0	7.8	7.9
	20	326	326	21.3	21.1	7.8	8.0
	100*	345	337	22.9	21.9	7.8	8.3
7	3	339		24.4		8.7	
	14	347	346				
	21	355	350			8.6	9.0
	37*	365	356	25.7	25.7	8.6	9.1
	80*	355	358	26.3	25.8		

\* Oxygen less than 20 per cent.

TABLE V.

*Molecular Concentration of Anions and Cations in Serum of Beef Blood in Equilibrium with 3 Per Cent Carbon Dioxide.*

Experiment No.	Na	K	Sum of the cations.	Chloride.	Bicarbonate.	Phosphate.	Sum of the anions.	Excess of cations.
4	0.1461	0.0053	0.1514	0.1112	0.0275	0.0024	0.1411	0.0103
5	0.1422	0.0054	0.1476	0.1090	0.0294	0.0027	0.1411	0.0065
6	0.1401	0.0053	0.1454	0.1068	0.0293	0.0025	0.1386	0.0068
7	0.1474	0.0062	0.1536	0.1076	0.0270	0.0028	0.1374	0.0162

Although we did not determine the sulfate, calcium, or magnesium of plasma, we venture to tabulate our figures for the other known inorganic cations and anions. De Boer (1917), considers the normal  $\text{SO}_4^-$  value to be about 20 mg. per 100 cc. which amounts to a molar concentration of 0.0021. Mean values for: calcium = 0.0027 M; magnesium = 0.0010 M. Normally, then, we have a considerable excess of cations which presumably in accord with L. J. Henderson (1908) and others are combined with the proteins. We have unaccounted for, except in this way, about 7 per cent of the bases of serum.

It is evident from the table that the chloride concentration is equal to about 75 per cent of that of sodium. We have found in our analysis of human blood that a marked deviation of chloride from the normal is accompanied by a corresponding change of sodium.

#### CONCLUSIONS.

*In vitro* experiments on beef blood equilibrated with various tensions of  $\text{CO}_2$  show the following points: (1) Equivalence of loss of chloride to gain in bicarbonate of serum. Though a migration of phosphate may occur, it is quantitatively of little importance in the transport of carbon dioxide; (2) non-transference of either sodium or potassium from cells to serum; and (3) a marked increase of corpuscle volume with increasing tensions of carbon dioxide.

#### PROTOCOLS.

Under the column headed,  $\text{CO}_2$ , we mean only the amount of carbon dioxide that was present in the 6 liters of gas passed through the saturators. The actual per cent of  $\text{CO}_2$  in the saturator did not nearly equal that of the entering gas. Unless otherwise stated this mixture contained between 20 and 21 per cent of oxygen. In Experiment 7 the amount of  $\text{CO}_2$  in the saturators was determined.

In order to diminish discrepancies due to analytical errors, artificial serums were employed in Experiments 1, 2, and 3. We had hoped to make the molar concentration of bicarbonate equal that of the chloride. This was unsuccessful. These data are included because of the close approximation of decrease of chloride to increase of bicarbonate.

## 390 Ion Migration between Cells and Plasma

The data in the protocols are used for the preparation of the tables. In each case the necessary corrections are applied as in the example given in Table II.

*Experiment 1.*—Defibrinated beef blood centrifuged and serum removed. An artificial isotonic serum, containing protein, glucose, potassium chloride, and bicarbonate was added. Equilibrated at 25°C. Considerable hemolysis in artificial serum. Serum bicarbonate by the Van Slyke-Cullen method.

Treatment of whole blood.	Corpuscles.	NaCl		NaHCO <sub>3</sub> concentration.
		Per 100 cc.	Concentration.	
<i>CO<sub>2</sub> per cent</i>	<i>per cent</i>	<i>mg.</i>	<i>mol</i>	<i>mol</i>
5	55.1	194	0.0332	0.0072
10	55.6	191	0.0327	0.0081
20	56.7	185	0.0316	0.0098

*Experiment 2.*—Defibrinated beef blood centrifuged and serum removed. An artificial serum, containing serum proteins, glucose, and disodium phosphate was added. Equilibration at 25°C. Plasma bicarbonate by the Van Slyke-Cullen method.

Phosphorus determination gravimetrically: the weighing of  $\text{MgNH}_4\text{PO}_4 \cdot 6\text{H}_2\text{O}$  as described by Jones (1916) being used.

Treatment of whole blood.	Corpuscles.	NaCl		NaHCO <sub>3</sub> concentration.	Na per 100 cc.	P	
		Per 100 cc.	Concentration.			Per 100 cc.	Concentration.
<i>CO<sub>2</sub> per cent</i>	<i>per cent</i>	<i>mg.</i>	<i>mol</i>	<i>mol</i>	<i>mg.</i>	<i>mg.</i>	<i>mol</i>
5	49.1	168	0.0287	0.0112	249	78.9	0.0254
20	50.0	164	0.0280	0.0126	253	80.5	0.0259

*Experiment 3.*—Defibrinated sheep blood. Whole blood centrifuged and serum removed. The same volume of an artificial isotonic serum, containing the serum proteins, glucose, and inorganic salts was added. The concentration of the sodium salts was made small in order to detect any part that its transfer might play in the attainment of equilibrium. Equilibration at 25°C. Considerable hemolysis occurred. Plasma bicarbonate by the Van Slyke-Cullen method.

Treatment of whole blood.	Corpuscles.	NaCl		NaHCO <sub>3</sub> concentration.	Na per 100 cc.
		Per 100 cc.	Concentration.		
<i>CO<sub>2</sub> per cent</i>	<i>per cent</i>	<i>mg.</i>	<i>mol</i>	<i>mol</i>	<i>mg.</i>
3	56.3	214	0.0366	0.0097	150
15	56.8	199	0.0340	0.0131	151

*Experiment 4.*—Defibrinated beef blood. Equilibration at 25°C. No hemolysis. Bicarbonate by titration.

Treatment of whole blood.	Corpuscles.	NaCl		NaHCO <sub>3</sub> concentration.	Na per 100 cc.	K per 100 cc.	P per 100 cc.
		Per 100 cc.	Concentration.				
CO <sub>2</sub> per cent	per cent	mg.	mol	mol	mg.	mg.	mg.
3	38.4	650	0.1112	0.0274	336	20.7	7.4
10	39.7	632	0.1081	0.0311	336		7.4
20	40.0	622	0.1063	0.0352	350	21.6	7.3

The volume change of corpuscles at 10 per cent CO<sub>2</sub> seemed to us peculiarly large in comparison with the 20 per cent. Upon the assumption that there is no exchange of nitrogen, we ran Kjeldahl determinations on the serum. The figures found follow.

CO <sub>2</sub> per cent	N per 100 cc. per cent
3	1.017
10	1.030

When these results were used to correct the chloride and bicarbonate figures we obtained the following results.

Gain in base by loss of HCl,  $0.1127 - 0.1081 = 0.0046$  M

“ “ “ as NaHCO<sub>3</sub>,  $0.0311 - 0.0278 = 0.0033$  M

The figures from our usual method of calculation are: by loss of HCl,  $-0.0055$  M; by gain of NaHCO<sub>3</sub>,  $+0.0031$  M.

*Experiment 5.*—Defibrinated beef blood. No hemolysis. Bicarbonate by titration.

Treatment of whole blood.	Corpuscles.	NaCl		NaHCO <sub>3</sub> concentration.	Na per 100 cc.	K per 100 cc.	P per 100 cc.
		Per 100 cc.	Concentration.				
CO <sub>2</sub> per cent	per cent	mg.	mol	mol	mg.	mg.	mg.
3	40.9	637	0.1090	0.0294	327	21.1	8.3
10	41.2	626	0.1071	0.0318	331	21.1	8.3
20	41.8	610	0.1043	0.0366	332	21.1	8.3
100	43.4	571	0.0977	0.0444	345	23.8	8.3

*Experiment 7.*—Defibrinated beef blood. No hemolysis. Bicarbonate by titration. Equilibrated with the following gas mixtures.

CO <sub>2</sub> per cent	O <sub>2</sub> per cent
3	20-21
14	20-21
21	20-21
37	13.0
80	4.0

Treatment of whole blood.	Corpuscles.	NaCl		NaHCO <sub>3</sub> concentration.	Na per 100 cc.	K per 100 cc.	P per 100 cc.
		Per 100 cc.	Concentration.				
CO <sub>2</sub> per cent	per cent	mg.	mol	mol	mg.	mg.	mg.
3	46.2	629	0.1076	0.0270	339	24.4	8.7
14	47.3	596	0.1020	0.0351	347		
21	47.9	584	0.0998	0.0380	355		8.6
37	48.8	570	0.0976	0.0408	365	25.7	8.6
80	49.0	561	0.0960	0.0431	355	26.3	

### Addendum.

We are indebted to Dr. Donald D. Van Slyke for calling our attention to a point in our paper which perhaps needs further explanation. The point concerns the fact that as stated on page 380 we have determined the shift of ions occasioned by different tensions of CO<sub>2</sub>, but in all cases have determined the bicarbonate by titration to an arbitrarily fixed and *constant* pH and have thus excluded from consideration the additional shift of base from serum proteins to carbonic acid which doubtless occurs with *changing* pH.

With Dr. Van Slyke's consent we append his comment on this point, with which we agree.

"There are two types of reactions with plasma by which increase in [H<sub>2</sub>CO<sub>3</sub>] causes increase in [BHCO<sub>3</sub>]; *viz.*,



the HCl being transported into the blood cells; and



(Van Slyke, 1921). Of these two reactions, the titration to the constant end-point measures only the first. The results therefore indicate that the above chloride reaction accounts for practically all the bicarbonate change due to migration of ions; *i.e.*, that Cl<sup>-</sup> is the only ion other than HCO<sub>3</sub><sup>-</sup> that plays a significant part in the acid-base shift between cells and plasma. The results do not indicate the relationship between the amount of BHCO<sub>3</sub> formed as the result of this migration and the amount formed by reactions with plasma buffers of the type exemplified above by Reaction (2)."



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## EXPERIMENTAL RICKETS IN RATS.

### II. THE FAILURE OF RATS TO DEVELOP RICKETS ON A DIET DEFICIENT IN VITAMINE A.\*

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PLATES 1 TO 3.

(Received for publication, May 28, 1921.)

The most recent hypothesis regarding the etiology of infantile rickets places it in the group of deficiency diseases resulting from a lack of vitamine. This hypothesis was advanced from a theoretical standpoint by Funk, and later was sustained by Mellanby as the result of experiments on dogs (1). The latter believes he has proved that when puppies are deprived of the so called fat-soluble vitamine (vitamine A) they develop rickets. The conclusions of Mellanby were accepted by the committee on accessory food factors of Great Britain (2) appointed by the Medical Research Committee and the Lister Institute.<sup>1</sup> This report considers the fat-soluble factor, or a factor with similar distribution, synonymous with the antirachitic factor, and presents a table of antirachitic foods of three grades of potency. In a previous paper one of the authors has shown that infants do not develop rickets when on a diet containing a minimal amount of this factor, but that on the contrary they not infrequently develop rickets in spite of receiving food rich in this vitamine, and concluded, therefore, that this unidentified factor could not be regarded as the antirachitic vitamine, and that it does

\* Read in abstract before the Society of Experimental Biology and Medicine, May 18, 1921.

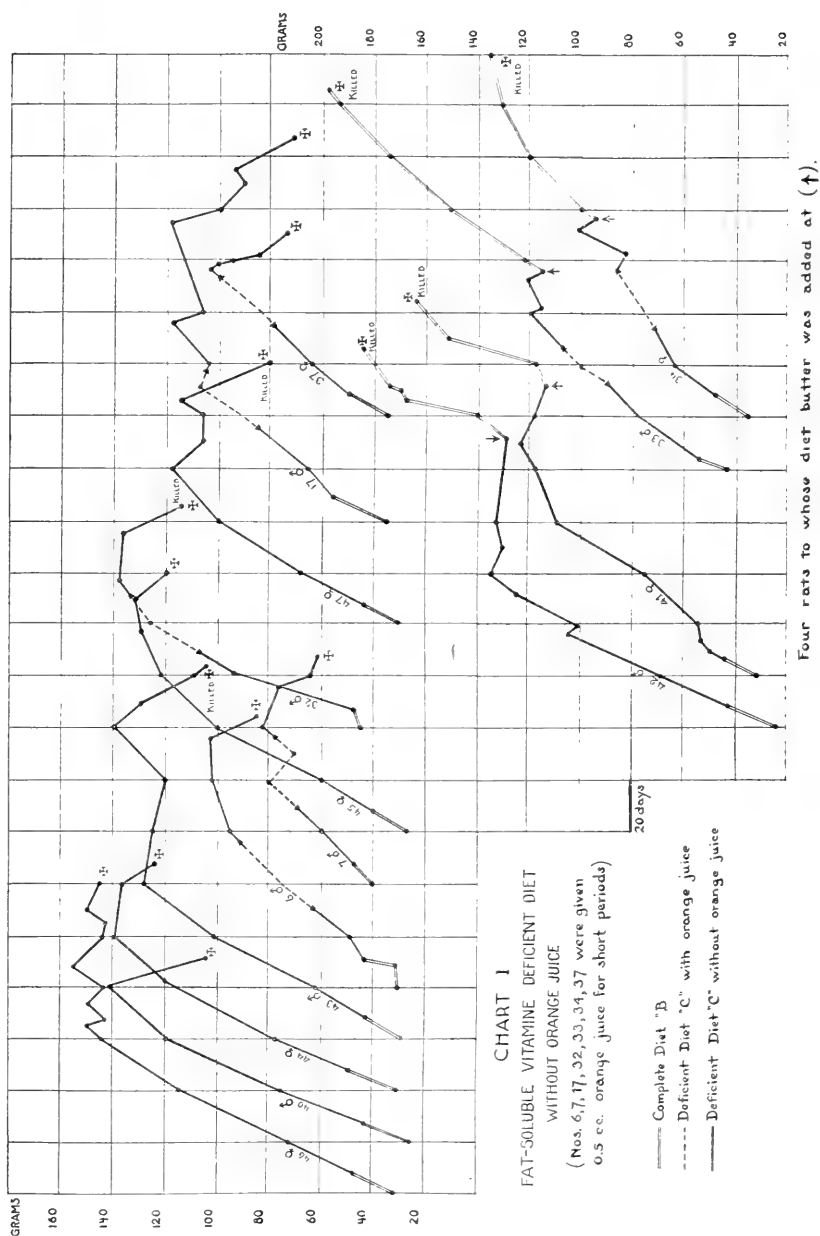
<sup>1</sup> Experimental evidence against the contention of Mellanby that the fat-soluble vitamine is the determining factor in rickets, has been presented by Paton, Findlay, and Watson (*Brit. Med. J.*, 1918, ii, 625), and by Paton and Watson (*Brit. J. Exp. Path.*, 1921, ii, 75).

not exert a controlling influence on the development of this disorder (3). More recently McCollum, Park, and their associates (4) have stated that in their experience rats fed on certain diets deficient in the so called fat-soluble A or in both that substance and calcium develop "a condition identical with the rickets of human beings." In a paper just published (5) they express the opinion that the fat-soluble A vitamine is not the sole cause of rickets, but that it cannot be excluded as an etiological factor in the production of this disorder.

The following investigation concerns itself with the effect on young rats of a diet markedly deficient in the fat-soluble vitamine. That lesions similar to those of infantile rickets can be induced in young rats has been shown recently by the work of Shipley and his associates (5) and by Sherman and Pappenheimer (6). For our purpose rats weighing about 30 gm. were placed on a basal ration (Diet C) similar to that employed by Drummond and Coward (7):

Casein.....	21 per cent
Rice starch.....	57 " "
Salt mixture.....	5 " "
Crisco.....	17 " "
Yeast.....	60 mg.

The casein was extracted with about twice its volume of cold alcohol by means of slow filtration which lasted 3 to 4 hours, until the last washings came through colorless. It was then extracted with ether (using about twice the volume of casein) for about 48 hours, and employing two changes of ether. The salt mixture was that used by Osborne and Mendel. Yeast was the Harris extract, prepared according to the formula of Osborne and Wakeman (8). One group of animals was also given orange juice, 0.5 cc. daily. Both the yeast and the orange juice were not incorporated in the food, but given separately, so as to make certain that they were consumed in full amount. In addition to this ration, which was complete except for an almost total lack of the fat-soluble vitamine, we employed Diet B, which was complete in all respects, 6 per cent of butter fat having replaced an equivalent amount of Crisco. In some instances Diet B was given practically throughout the experimental period, in order to allow of a comparison of growth and health between



a complete ration and one deficient in the fat-soluble vitamine (Chart 3); in other instances Diet B was substituted where the deficient diet had been maintained for months (Chart 1).

The accompanying charts and tables illustrate our results. It will be seen that the rats on this deficient diet failed to gain in weight after a period of about 2 months, that somewhat later they began to lose in weight, and that they invariably died prematurely. This is clearly illustrated by the graphs of Charts 1 and 2. The corresponding tables (Tables I and II) show that these rats in almost all instances showed lesions of keratitis, which is considered distinctive of this vitamine deficiency. These are the two criteria which are accepted as pathognomonic of a lack of the fat-soluble factor, growth failure and keratitis, so that this diet must be judged to have been markedly lacking in this essential factor. That such was the case is evident from a survey of Chart 3, which differs from the preceding groups (Charts 1 and 2) merely in that butter was added to the dietary. Here growth invariably was excellent. This distinction is well exemplified also in Chart 1 and Table IV, which show the sharp rise in the growth curve and the rapid cure of the ophthalmia when 6 per cent of butter was substituted for an equal amount of Crisco.

One group of rats on the deficient dietary was given 0.5 cc. of orange juice daily, whereas the other group received no antiscorbutic foodstuff. These groups were constituted in order to determine whether a lack of antiscorbutic still further retarded growth and favored the development of the eye lesions. As is well known, opinion is divided as to whether rats require antiscorbutic vitamine or whether they thrive normally when deprived of this factor. On adding orange juice a definite and prompt increase in weight was brought about in some instances, but this gain did not persist for long periods. In general, it may be stated that the curves in Chart 2, representing rats which received orange juice do not appear superior to those in Chart 1, where this antiscorbutic was lacking. Ophthalmia occurred less frequently and with less severity among the rats which received orange juice. This was more noticeable clinically than on pathological examination; among the eleven rats which received no orange juice, only one failed to develop ophthalmia during life (Table I),

TABLE I.  
*Fat-Soluble Vitamine Deficient Diet, No Orange Juice.*

Serial No.	Days on deficient diet.	Eyes during life.	Infections during life.	X-ray.	Pathological examination.		
					Rickets.	Ophthalmia.	Other lesions.
6	113	Purulent exudate and puffiness.		Negative 111th day.	None.*	Slight keratitis.*	Normal.
7	80	Purulent exudate.	Diarrhea.		"*	No keratitis.*	Hemorrhagic colitis; cestode infection.
17	139	"			"*	Minimal lesion.*	Focal abscesses in kidney. Diphtheritic gastritis.*
32	77	"			"*	Corneal ulcer; keratitis.*	Acute tracheitis.*
37	65	Clear.	Diarrhea.		"*	None.*	Intestines contain thin fluid and gas.
40	63	Puffiness.	Bloody urine.		"*	Conjunctivitis.*	Pyelitis (L); bronchopneumonia.*
43	138	Purulent exudate; puffiness.	Diarrhea.	Negative 137th day.	"*	Mild keratitis.	Suppurative infection of lung and submaxillary gland. Intestinal ectodes.
44	82	Bloody exudate.			"*	Minimal lesions.*	Pyelitis (L).
45	96	"			"*	Conjunctivitis.	" (double).
46	116	"	Purulent urine.		"*	Very slight keratitis.*	
47	90	Bloody exudate.			"*	No keratitis.*	None.

\* Confirmed by microscopic examination.

TABLE II.  
*Fat-Soluble Vitamine Deficient Diet, with Orange Juice.*

Serial No.	Days on deficient diet.	Eyes during life.	Infections during life.	Pathological examination.		
				Rickets.	Ophthalmia.	Other lesions.
2	119	Slight exudate.	Ulcer under jaw.	None.*	None.	Healing ulcer in neck.
3	115	Clear.	Infected ear; rhinitis.	" "	Slight keratitis.*	Parasitic cysts in renal pelvis.
5	151	Exudate and puffiness.		" "	" opacity of cornea.	Suppurative pneumonia.*
9	119	Clear.	Diarrhea; rhinitis.	" "	Slight keratitis.*	None.*
11	91	"	Otitis media.	" "	" "	Purulent otitis (L).
12	154	Purulent exudate and puffiness.	Diarrhea.	" "	" "	Submaxillary abscess; abscesses of lung.
13	105	Clear.	Rhinitis.	" "	No keratitis.	Parasitic infection of renal pelvis.
16	169	Purulent exudate and puffiness.		" "	Slight keratitis.*	None.
21	115	Clear.		" "	" "	Bronchiectasis.*
22†	182	Purulent exudate; puffiness.		" "	" "	
27	108	Bloody exudate.		None.*	" infiltration of cornea.*	None.
28	59	Clear.	Diarrhea.	" "		
31	53	Bloody exudate.		" "	Conjunctivitis.	Intestinal cestodes.*
36	54	Clear.	Otitis media.	" "	Very slight keratitis.*	Focal necroses in liver.*

\* Confirmed by microscopic examination.

† X-ray, negative, 182nd day. Not killed.



TABLE III.  
*Long Period on Deficient Diet, Followed by Addition of Butter, with Orange Juice.*

Serial No.	Total days on diet.	Period on deficient diet. <i>days</i>	Eyes during life.		Infections during life.	Pathological examination.		
			While on deficient diet.	While receiving butter.		Rickets.	Ophthalmia.	Other lesions.
8	210	172	Clear.	Clear.	Diarrhea.	None.	None.	None.
14	202	169	Bloody exudate one day, (145th).	"	"	"	"	Intestinal cestodes.
24	202	169	Purulent exudate on 144th to 169th day.	Cured in 4 days.	Pus in urine.	"	"	Vesical calculi.
33	145	74	Clear.	Clear.	Diarrhea.	"	"	
34	145	70	"	"	"	"	"	
41	135	104	Bloody exudate 97th to 104th day.	Cured in 3 days.	"	"	"	None.
42	135	104	Bloody exudate and puffiness, 95th to 104th day.	" " 2 "	"	"	"	"

whereas among the fourteen which did receive an addition of orange juice, in seven the eyes were at all times normal (Table II).

In this connection it should be borne in mind that some preliminary observations of Osborne and Mendel (9) indicated that orange juice contains traces of the fat-soluble vitamine.

✓ Probably a deficiency of the antiscorbutic factor intensifies the alteration of the cells brought about by other nutritive deficien-

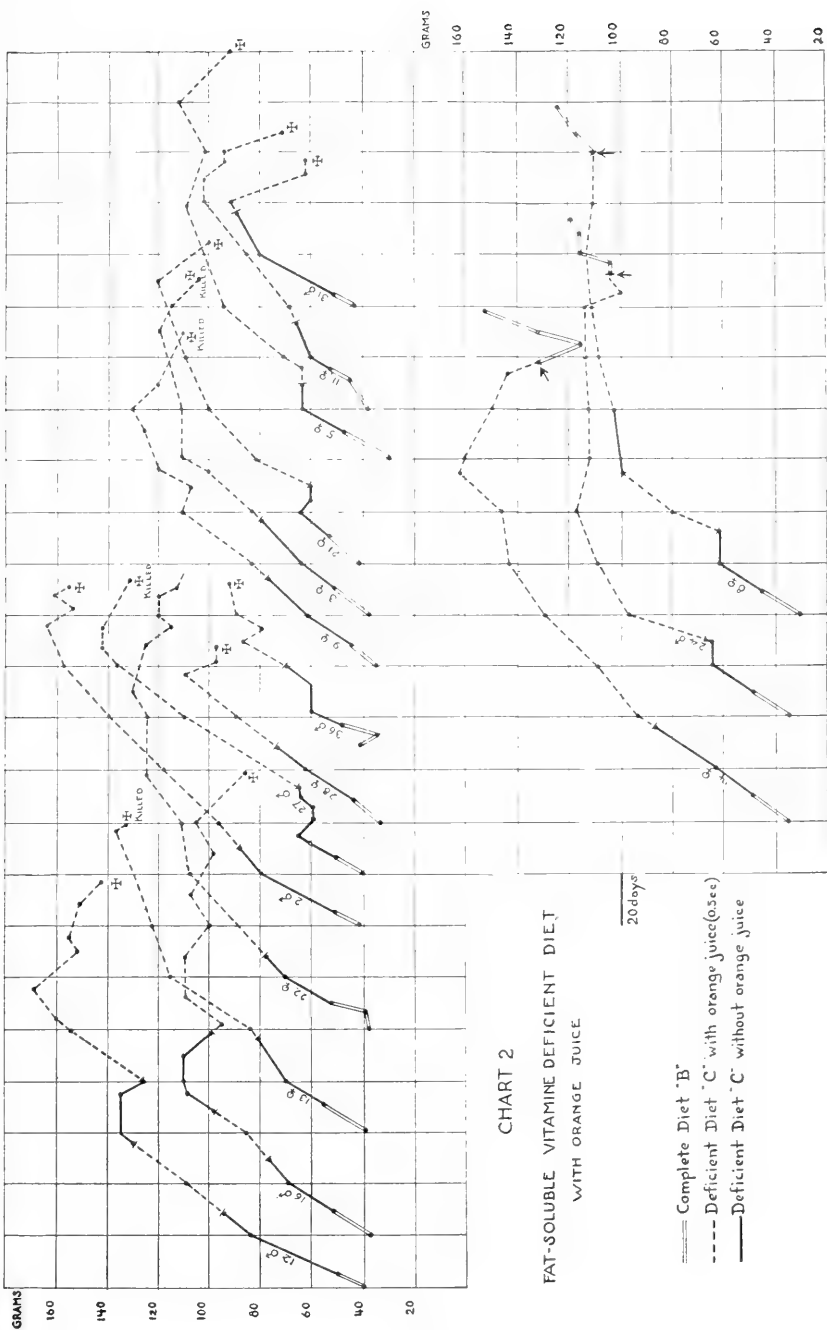
TABLE IV.

*Control Rats on Full Diet for Entire, or Almost Entire, Period.*

Serial No.	Total days on diets.	Period on deficient diet.	Eyes during life.	Infections during life.	Pathological examination.		
					Rickets.	Ophthalmia.	Other lesions.
With orange juice.							
15	114	11th to 33rd day.	Clear.	Rhinitis.	None.	None.	None.
18	148	0	"		"	"	"
20	148	0	"		"	"	"
25	179	0	"		" *	" *	"
26	131	6th to 30th day.	"		"	"	"
38	114	0	"		"	" *	"
48	137	0	"		?	" *	"
Without orange juice.							
1	121	11th to 44th day.	Slight exudate on 89th day.		None.	None.	None.
10	117	11th to 44th day.	Clear.		"	"	"
35	114	6th to 42nd day.	"		"	"	"

\* Confirmed by microscopic examination.

cies. In experiments on scurvy in guinea pigs ophthalmia and keratitis have been observed by us. Similar lesions have been reported in the course of human scurvy (10). In this connection it may be mentioned that conjunctivitis, unaccompanied by puffiness of the lids, occurs not infrequently among white rats, especially where they have not been kept in the dark.



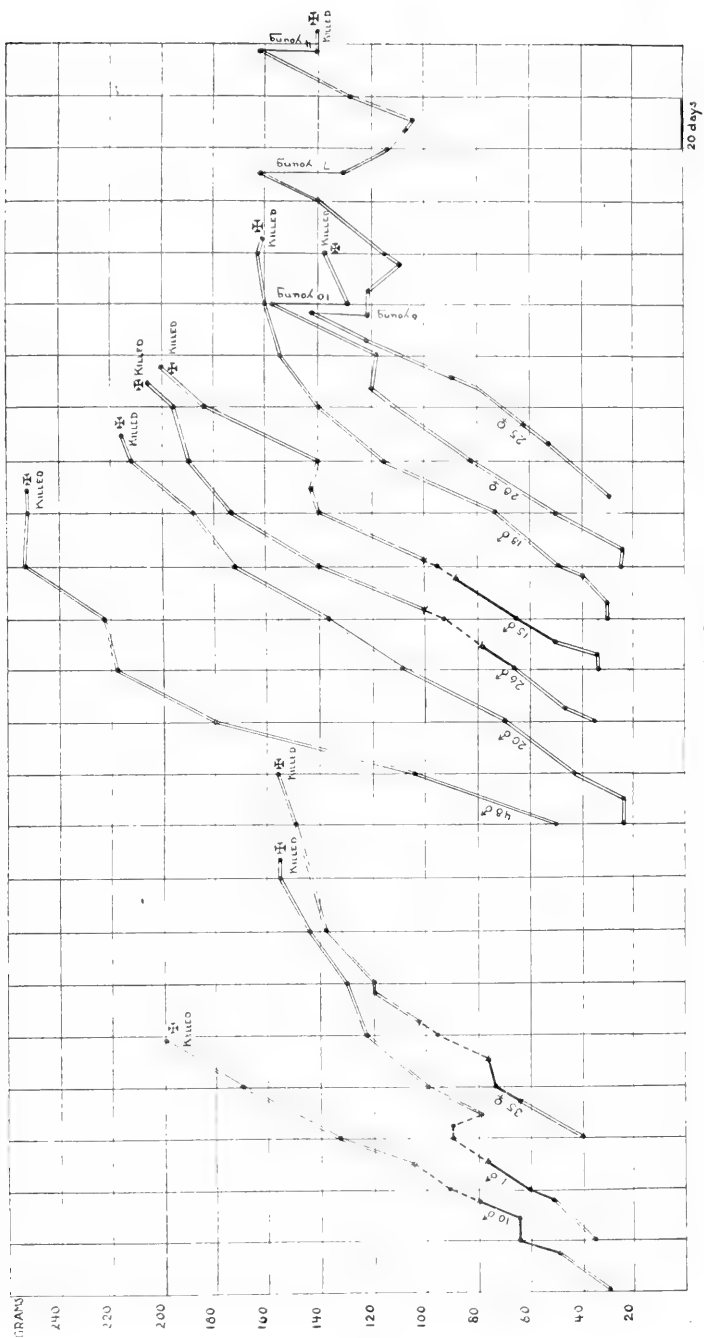


CHART 3  
FAT-SOLUBLE VITAMINE ADEQUATE DIET "B"

*Pathological Examination.*

As regards gross changes the skeletal system was found normal in all respects. In no instances were there the deformation of the thorax, the beading and angulation of the costochondral junctions, the multiple infractions surrounded by masses of callus, which form so striking a feature of rickets in rats. The long bones also gave evidence of no undue pliability, curvature, or epiphyseal enlargement. Under the microscope,<sup>2</sup> however, certain deviations from the normal picture were found quite regularly. As will be evident from the accompanying description and illustrations, these lesions were in no way suggestive of rickets but were correlated rather with the deficient growth of the animals.

The zone of proliferating cartilage was narrow; the columns rarely exceeded four cells in depth, and frequently were composed of not more than two or three cells. The matrix of the cartilage in this zone of proliferation was invariably densely calcified, not only the capsules of the cartilage cells but the tissue between them taking a deep purplish blue stain indicative of calcium. This was confirmed in some of the preparations by applying von Kossa's silver nitrate method.

The most striking departure from the normal was found in the subchondral zone. Instead of a series of stout trabeculae showing an orderly parallel arrangement about the calcified cartilage matrix, and directed in the long axis of the rib, the primary spongiosa was defective. In many cases it was represented by two or three delicate and irregularly disposed trabeculae leaving the calcified cartilage exposed over a large portion of the chondrocostal line; in others there was a continuous thin transverse plate of fully calcified bone, limiting the cartilage and fusing with the calcified matrix. The osteoblasts, surrounding these trabeculae or lying against the subchondral bone plate, were inconspicuous in contrast to their swollen appearance in actively proliferating bone. Osteoid tissue was conspicuously absent here as also along the completely calcified cortical layer.

<sup>2</sup> After fixation in Muller formol, ribs were decalcified for 5 to 15 days in Muller's fluid, embedded in paraffin and stained with hematoxylin eosin. The examination of the long bones has not yet been completed.

The trabeculae of the secondary spongiosa were often better developed, having been formed during the period of more active growth. The marrow usually was of the normal cellular type.

TABLE V.

*Histological Picture of Costochondral Junctions of Rats Fed Complete and Incomplete Diets.*

	Complete diet.	Fat-soluble A deficient diet.	Rachitic diet.
Zone of proliferating cartilage.	2-4 cells deep. Regular arrangement. Matrix calcified.	1-4 cells deep. Columns often slightly separated. Matrix calcified.	1-20 cells deep. Irregular prolongations into metaphysis. Matrix uncalcified.
Primary spongiosa.	Stout trabeculae corresponding to columns of cartilage cells, parallel alignment.	Trabeculae reduced in number and size, irregular in arrangement, often fused into thin plate, completely calcified.	Composed chiefly of osteoid, with core of calcified cartilage or bone. Dense tissue with narrow vascular spaces.
Secondary spongiosa.	Well developed.	Well developed in some cases, deficient in others.	Dense trabeculae with wide osteoid margins.
Cortex.	Narrow osteoid margin in young growing rats; invisible in older animals.	No visible osteoid margin. No infractions.	Irregular broad subperiosteal and endosteal osteoid margins. Frequent infractions, with masses of cartilaginous and osteoid callus.

In some rats which had acute suppurative infections in other tissues (submaxillary gland, kidneys), there was an unusual proportion of polymorphonuclear leucocytes among the marrow ele-

ments. In a few preparations the marrow immediately about the cartilage was depleted of blood-forming elements, and composed predominantly of pale polyhedral cells, often forming multinucleated masses about the cartilage.

The features which differentiate the bones of these animals maintained on fat-soluble vitamine deficient diet from those of rats on an adequate diet and pursuing normal growth as well as well as from those with rachitic lesions, are brought out in Table V.

From the foregoing comparison it is clear that the skeletal changes in rats on a fat-soluble deficient diet are in no respect suggestive of rickets, but may be interpreted as due to an inactive osteogenesis. This is what might be expected in view of the stationary or declining weight and arrested growth.

The ocular lesions have been so thoroughly studied by Stephenson and Clark (11), and by Wason (12) that detailed reference need not be made to our experiences. The histological changes were such as have been described by these observers. Of the 25 rats comprised in Tables I and II it will be noted that in 5 the corneæ were normal on microscopic examination.

Incidental infections were encountered in a large proportion of the rats; notably, submaxillary abscesses, suppurative infections of the urinary tract, and bronchiectases. Infestation with the cestode, *Hymenolepis murina*, was also extremely common. Vesical calculi, described by Osborne and Mendel (13) in rats deprived of the fat-soluble vitamine, were encountered at autopsy in one instance (Rat 24). In this animal it was associated with pyelitis. Its occurrence in this connection is probably due in part to the susceptibility to infections induced by a diet lacking in this vitamine.

#### CONCLUSION.

Young rats receiving a diet complete except for a lack of the fat-soluble vitamine invariably failed to grow and generally developed keratitis. The keratitis developed less frequently when the ration included orange juice. If this diet is continued for a period of months the animals die, either of inanition or, more often, of some intercurrent infection. The skeletons of such rats show no gross changes whatsoever. Microscopic examination

of the bones of 22 rats on a ration of this character presented definite signs of a lack of active osteogenesis, but in no instance lesions resembling rickets. In view of these results and their conformity with our previous experience in regard to infantile rickets, we are of the opinion that this vitamine cannot be regarded as the antirachitic vitamine, and that, if the diet is otherwise adequate, its deficiency does not bring about rickets.

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## EXPLANATION OF PLATES.

## PLATE 1.

FIG. 1. Normal rib. Rat 82. Weight 158 gm. 52 days on complete diet; continuous growth and gain in weight. Decalcified 3 days in Muller's fluid.

FIG. 2. Rib of Rat 17. 139 days on fat-soluble A deficient diet. Maximum weight 118 gm. Terminal weight 71 gm. Decalcified 10 days in Muller's fluid.

Zone of preparatory calcification is very narrow, not exceeding three cells in depth. Cartilage cells separated by densely calcified plate. Primary spongiosa represented by continuous bony plate, without definite trabeculae. Cortex thick, without visible osteoid margin. Osteoblasts inconspicuous.



## PLATE 2.

FIG. 3. Rib of Rat 44. 82 days on fat-soluble A deficient diet. Maximum weight 140 gm. Terminal weight 125 gm. Decalcified 5 days in Muller's fluid.

Zone of preparatory calcification not over four cells in depth, regular, matrix completely calcified. Osteogenesis at epiphysis is fairly active. Spongiosa composed of coarse trabeculae, with absent or inconspicuous osteoid margin. Cortex broad and completely calcified. Marrow cellular, not fibrous.

FIG. 4. Rib of Rat 58. 34 days on rickets-producing diet. Marked rachitic lesions. Note increased width and irregularity of proliferative cartilage, absence of calcium deposition, great excess of osteoid in region of metaphysis and about cortex. Decalcified 5 days in Muller's fluid.

## PLATE 3.

FIG. 5. Rat 16. 175 days on fat-soluble A deficient diet. X-ray. Absence of rachitic lesions at upper epiphysis of tibia.

FIG. 6. Rat 59. 41 days on rickets-producing diet. X-ray, showing rachitic changes in upper epiphysis of tibia.





FIG. 1.

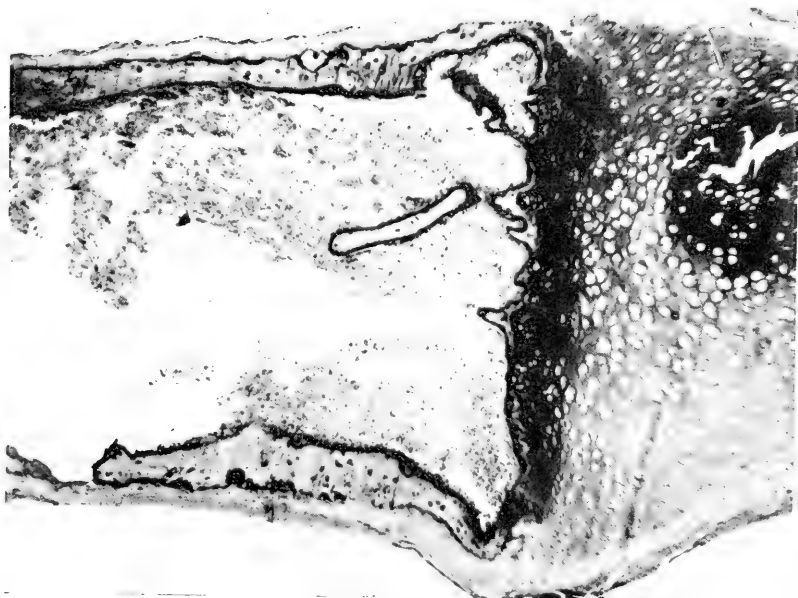


FIG. 2.

(Hess, McCann, and Pappenheimer: Experimental rickets in rats. II.)



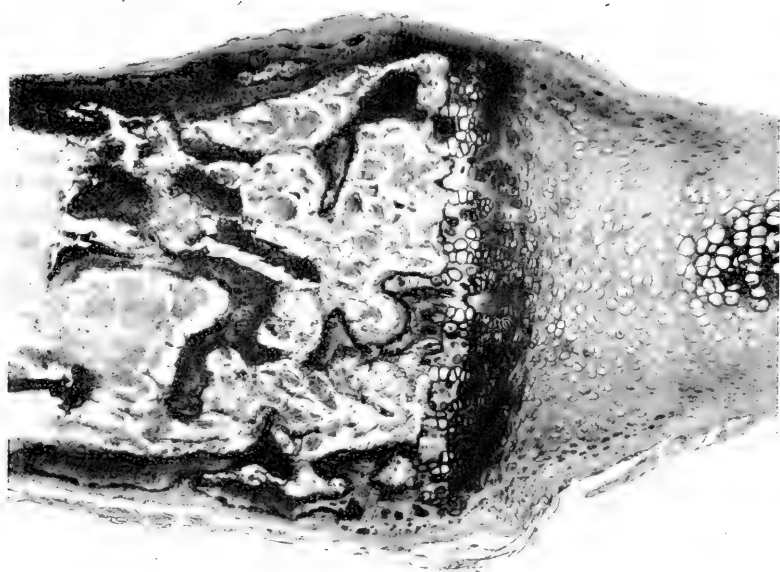


FIG. 3.

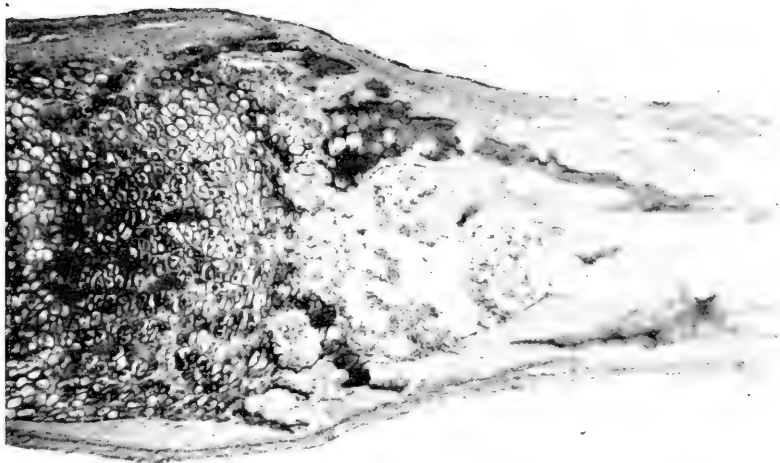


FIG. 4.

(Hess, McCann, and Pappenheimer: Experimental rickets in rats. II.)





FIG. 5.



FIG. 6.

(Hess, McCann, and Pappenheimer: Experimental rickets in rats. II.)





**METHODS OF EXTRACTING AND CONCENTRATING  
VITAMINES A, B, AND C, TOGETHER WITH AN  
APPARATUS FOR REDUCING MILK, FRUIT  
JUICES, AND OTHER FLUIDS TO A  
POWDER WITHOUT DESTRUCTION OF VITAMINES.**

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(Received for publication, May 16, 1921.)

I.

As the population increases, the question of food supply becomes more urgent, and the vitamins being necessary food constituents need to be conserved. It is therefore important to develop methods for extracting them from plants that are not considered edible and for preserving them in foodstuffs that require storage. Except for the perfection of the drying apparatus, the methods here described, were worked out in 1919-20. Methods A, B, and C, are especially devised for the vitamins, respectively A, B, and C. Each product is, in reality, a mixture of vitamins with one predominating. By these methods the vitamins are extracted with very little loss, and concentrated without the use of very expensive reagents.

The unique features of the methods are believed to be; first, the extraction of vitamin A from green leaves or fruit skins by the use of high pressure (after moistening with alcohol). Second, the separation of the resinous and lipid material from the water-soluble portion in extracting vitamin B by increasing the H ion concentration up to the isoelectric point of these colloids; and third, the removal of the sugars from the B and C extracts by fermentation with baker's yeast. All processes are carried out in the absence of oxygen, and the drying is done very quickly.

In making the A extract, green leaves or fruit skins may be used. These are dried, preferably in the absence of oxygen,

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and then ground to a powder. This powder is moistened with alcohol by being thrown into a container half filled with boiling 95 per cent alcohol and allowed to remain without application of heat for 24 hours. The mass is then placed in a strong canvas bag and subjected to a pressure of 5,000 pounds to the square inch in a suitable press. The press-cake may be ground in a mill and reextracted in the same manner. The press-juice is dried in the apparatus described below. If, however, it is desired to recover the solvent, a preliminary concentration may be done in a vacuum pan. The product may be a sticky powder when absolutely dry, but absorbs water from the air, and becomes pasty. It contains resinous or fatty substances. It is of a waxy consistency when made from spinach and absolutely dry, but is hygroscopic.

The preparation of the water-soluble B is made from wheat germ or other foodstuff, such as yeast, which is rich in this vitamin. The wheat germ is treated in the same manner as the green leaves up to the pressing out of the extract, except that 80 per cent alcohol is used. The press-cake is ground in a mill and extracted again in the same manner. The press-juices are concentrated in a vacuum pan to about one-tenth of the original volume or until the first precipitate starts to form. An equal volume of water is now added and hydrochloric acid is added slowly and with stirring until a bulky and sticky precipitate forms. This is filtered off and the precipitate washed with distilled water and the washings are added to the filtrate. The precipitate is dried and extracted with benzene in any convenient extraction apparatus and the benzene is evaporated. The residue contains some of the wheat oil. The filtrate is brought to pH=4 to 5 and is fermented until the reducing sugar is lowered to about 1 per cent of its original value. The yeast is filtered off and added to the next batch of wheat germ and the filtrate is evaporated to dryness. In both the filtrate and the benzene extract, the water-soluble B is in quite concentrated form and there is practically no loss except the portion absorbed by the yeast and which would be recovered in the next batch.

The water-soluble C is extracted from fruits or tomatoes. If oranges are used they are pressed and the juice is run into vessels previously filled with carbon dioxide so as to exclude the

oxygen of the air, and baker's yeast added and a cover kept on so that no air enters. The fermentation is allowed to proceed at room temperature until the reducing sugar is lowered to about 1 per cent of its original value which requires about 24 to 48 hours. The juice is then filtered with the exclusion of air and is condensed by spraying (in the apparatus described below) in the absence of oxygen, until the volume is reduced to about one-twentieth of the original volume. It is then thrown into four volumes or more of 95 per cent alcohol and the precipitate thus formed is filtered off and the filtrate sprayed and reduced to dryness with the same precautions.

These three preparations may be designated A, B, and C. Preparation A contains sufficient fat-soluble A vitamine so that about 0.05 to 0.1 gm. daily added to a ration free from fat-soluble A will produce normal growth in a rat. It also contains considerable water-soluble B.

Preparation B is very rich in water-soluble B vitamine so that a very small dose of either the lipid or water-soluble fraction will cure a pigeon of polyneuritis. The lipid fraction will revive the hypodynamic heart of the turtle, but the water-soluble fraction is ineffective. Both fractions are effective growth stimulants.

Preparation C contains sufficient antiscorbutic vitamine so that about 5 to 10 cg. a day will prevent scurvy in a guinea pig on a basic diet supposedly free from water-soluble C for a period of about 2 months.

Preparation C was in one instance purified further by the removal of citric acid, but since it is highly hygroscopic and deteriorates more rapidly (when moist) when not acid, further work on this preparation was postponed awaiting the perfection of the drying apparatus.

The tests for the concentration of vitamins were made on rats, guinea pigs, and pigeons. The rats and guinea pigs were kept in metabolism cages, one unit of a 10 compartment cage being shown in Fig. 1. In case metabolic data were not kept, the pan and beaker under the cage were removed and a newspaper placed under the cage to catch the excreta. Drinking water was always present in the bottle. The basic diet for the guinea pigs was equal parts of white flour and alfalfa meal, and for the rats

was 10 per cent vitamine-free casein, 6 per cent dehydrated sea water, and 84 per cent white flour. In case vitamine A was investigated the rat diet contained 5 per cent dry Fleischmann's yeast and in case vitamine B was investigated the rat diet contained 1 gm. of butter fat per day.<sup>1</sup>

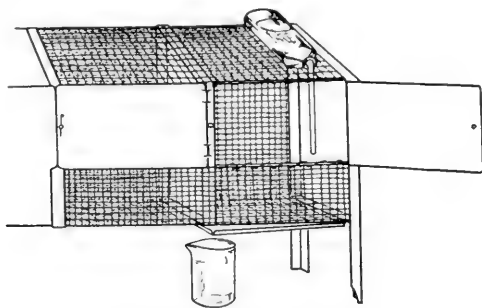


FIG. 1.

## II.

*The Drying Apparatus.*

Many forms of drying apparatus were tried, such as evaporation at low temperature in a rotating drum by a blast of air, vacuum pans through which a small stream of carbon dioxide and nitrogen passed, atomizer sprays in flue-gas, and evaporation in front of an electric fan at room temperature. None of these methods was efficient, and, therefore, the following method was devised.

The apparatus, Fig. 2, consists of a chamber near or at the bottom of which is the flue-gas exhaust, and in the center of the ceiling of which is the flue-gas and spray intake. The spray or distributor is rotated on the same shaft with the fan propelling the flue-gas at a speed of 5,000 revolutions a minute by an electric motor. The milk or other liquid passes down from a tank through a cock which regulates its flow into the hollow shaft of this apparatus. The furnace is built on top of the chamber.

<sup>1</sup> Some of the tests were made by Dr. M. A. Shillington, Helen Brenton, H. D. Reineke, and E. Dunlap, and these workers may publish details of their tests elsewhere.

It is constructed of fire-brick plastered with fire-clay, and has two flues that may be closed with valves, one extending upward, and the other closed by nichrome wire gauze and extending downward through an opening in the center of the ceiling. The apparatus propelling the flue-gas and making the spray is shown in

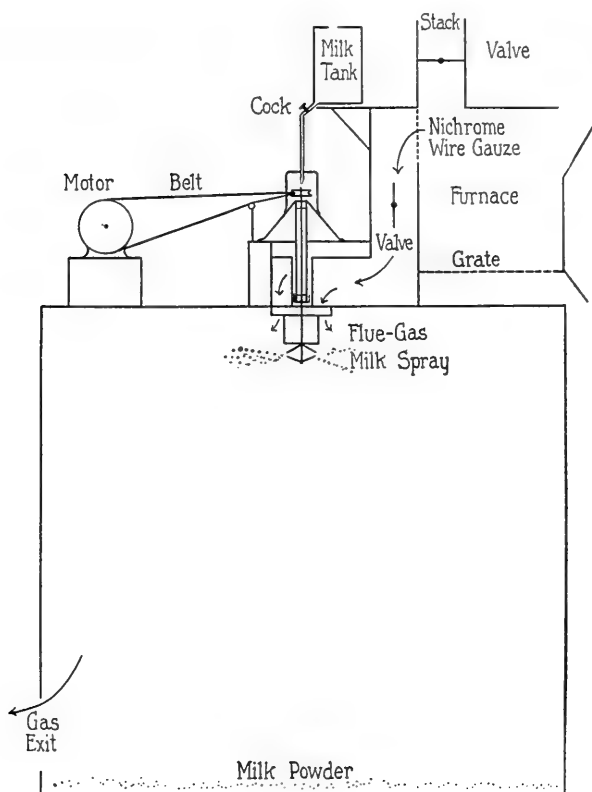


FIG. 2.

detail in Fig. 3. It revolves on a steel tube that serves as a shaft and which is driven by a pulley at the top. Inside this steel tube is an aluminum or brass tube, which touches the steel tube only at the extreme top and bottom. Between the steel tube and the aluminum tube is an air space to prevent the passage of heat

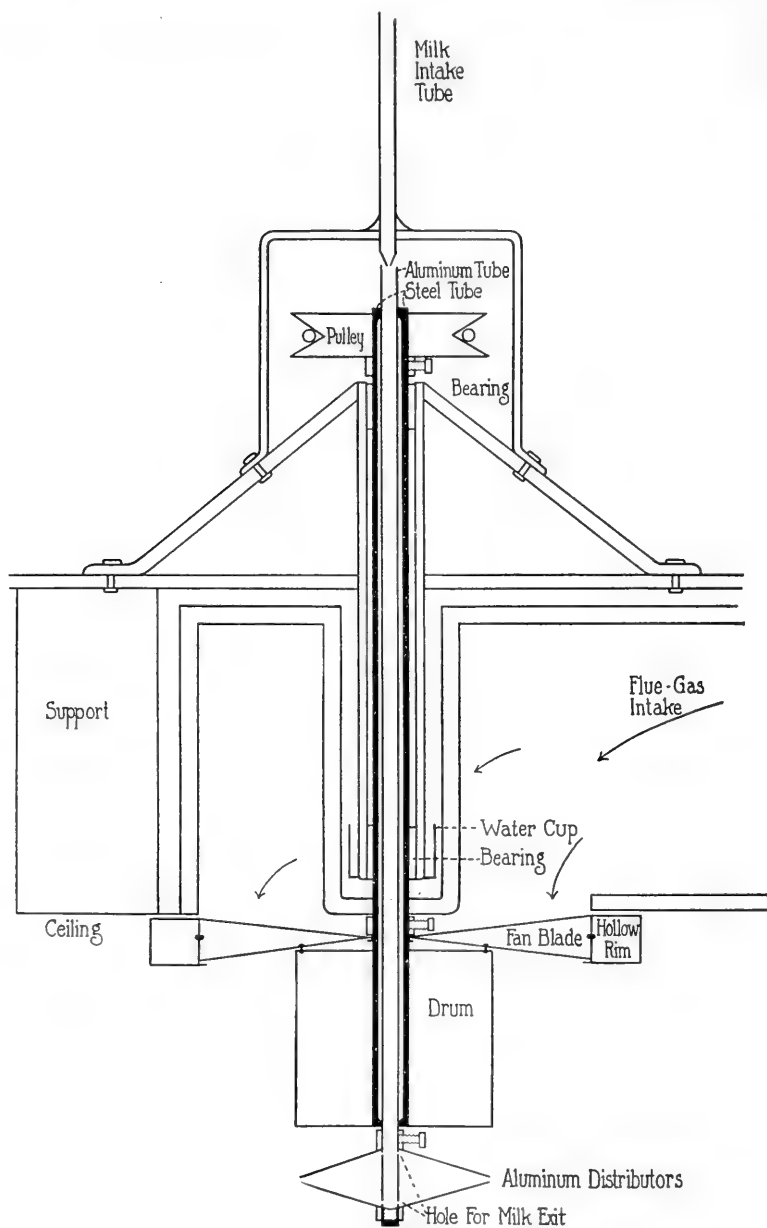


FIG. 3.

into the milk or other liquid being sprayed. Above the aluminum tube is fixed a nozzle which spurts a stream of the milk into the aluminum tube. The milk passes down the aluminum tube by gravity and out through a series of holes bored through the aluminum tube near the bottom, whence it strikes the surface of two cone-shaped aluminum distributors and spreads out on their surfaces and is thrown off from the periphery, which is cut into numerous saw-tooth-like points. From each point drops of liquid are thrown off by the rotation of the apparatus, producing a fine spray. On the steel tube, just above the aluminum distributors is a hollow drum which serves to prevent eddy cur-

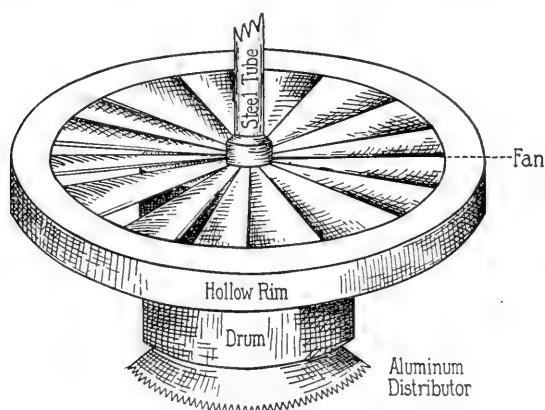


FIG. 4.

rents and retard passage of heat to the steel tube. Above the drum is a fan with a hollow rim. This fan forces the flue-gas downward, cutting the spray at right angles and rapidly evaporating the water. In order to prevent undue heating of bearings in which the steel tube rotates, an air space is provided between the flue-gas intake and the stationary tube in the ends of which the bearings are located. Also a deep groove or "cup" filled with water is provided on the lower end of this stationary tube. The aluminum distributor and fan are shown in perspective in Fig. 4. The hollow rim of the fan is useful to prevent burning of some of the spray that might be drawn against the rim of the fan by eddy currents. The portion of the flue right above the

fan is made of wire screen plastered on both sides with asbestos furnace cement. In order to prevent the presence of oxygen in the flue-gas the amount of air must be reduced to a point at which some carbon monoxide persists. This carbon monoxide will form a crust of carbonyl on metallic surfaces, which will peel off and drop in the chamber. It is, therefore, best to have all metal coming in contact with the heated flue-gas made of nichrome, which seems more resistant than other metals or alloys, and the amount of metal should be reduced as much as possible. Therefore, all stationary metal parts exposed to flue-gas are covered with asbestos furnace cement. In operating the apparatus the furnace is filled with fairly large pieces of charcoal or coke, and this is ignited by means of illuminating gas. The valve in the flue or stack that extends upward is opened until the heat of the furnace is sufficient to drive off volatile substances in the fuel. The valve in the stack is then closed and the valve in the flue extending downward into the chamber is opened and the motor is set going, thus driving the hot flue-gas into the chamber. When the chamber is heated to about  $150^{\circ}$  at the top a little water is passed downward through the aluminum tube in order to cool it. Then the fluid from the tank is allowed to pass slowly into the aluminum tube. The rate of flow of this fluid is regulated by the cock so that the temperature at the bottom of the chamber remains at about  $80^{\circ}$ . The temperature is greater at the top than at the bottom, and, if the walls are cooled, a condensation of moisture will take place within the chamber. It is therefore desirable to have the walls of non-conducting material, or else have the space surrounding these walls filled with the exhaust gas. It is desirable to have a series of thermometers with bulbs extending through the wall into the chamber at intervals from the top to the bottom. By reading these thermometers the flow of the fluid can be so regulated that the spray will always be reduced to a powder at the lowest possible temperature. The incoming flue-gas may be about  $1,000^{\circ}$ , but is instantly cooled by coming in contact with the spray. It is necessary, however, for the temperature within the chamber to be high enough to prevent recondensation of the moisture evaporated. Just what temperature that will be depends on the exact temperature of the flue-gas before meeting the spray and on whether there is any



loss of heat through the walls or floor of the chamber. The temperature must, in any case, be higher than that required by warm-air-drying systems, but this disadvantage seems to be compensated by the greater stability of the vitamine in the absence of oxygen. In case milk is sprayed the absence of oxygen prevents the oxidation of the fat.

As to how well this apparatus will work on a very large scale needs to be determined, but as an experimental apparatus, it has the great advantage that the cost of installation is comparatively small and changes are easily made. The necessity of steam boilers, steam radiators, and condensers is obviated. The greatest loss is the heat that passes out in driving off the volatile constituents of the fuel before starting the spray, but this heat could be used for other purposes. Also the exhaust gas, if not saturated with moisture may be used for drying vitamine-containing products on tray dryers. So far this apparatus has been used for drying the vitamine extracts described above and also for drying orange juice and milk.

The dimensions of this apparatus as it now stands are as follows: The chamber is 8 feet square and 12 feet high, the aluminum distributor is 3 inches in diameter, and the fan 6 inches in diameter inside the rim. The flue is also 6 inches in diameter. A  $\frac{1}{4}$  horse power motor is used. The size of the motor could be greatly decreased by the use of ball bearings replacing the Babbit metal bearings in which the steel tube rotates. The main difficulty experienced has been the burning of some of the spray around the edge of the flue-gas intake. Practically no spray sticks to the walls if the temperature is maintained high enough. In case it is desired merely to condense the spray, there is considerable condensation of moisture on the walls, and care should be taken that this does not drip into the condensed spray which falls into enamelled pans on the floor. Very little dust in the form of ashes need pass into the chamber if the apparatus is operated properly. The fan acts as a centrifugal dust separator. Two methods have been tried to improve the dust separation. One is the passage of gas through an asbestos filter, and the other is the modification of the fan so that it forms a more efficient centrifugal dust separator. The latter method is open to the objection that the dust accumulating in the fan must be periodically

removed or else it may throw the fan out of balance. The complete combustion of the fuel in the furnace is desirable. Apparently the nichrome wire gauze acts as a catalyzer when heated red hot. It is, however, necessary to have the bed of coals deep enough so that some carbon monoxide is formed in order to obviate the danger of an excess of oxygen. Apparatus for the analyses of flue-gas might be installed if the drying apparatus were used on a large scale.

While operating the apparatus as described above, the powder is kept at about  $80^{\circ}$  until the end of the operation. If it is desired to keep the powder cool, the flue-gas outlet may be made a few feet above the bottom, and the chamber below this outlet kept filled with cold  $\text{CO}_2$  or other gas. The flue-gas escaping from the outlet may be cooled and dried and used to fill the lower portion of the chamber. If this cool dry flue-gas is warmed slightly it may be used to complete the drying of the spray in case this was not accomplished by the time the spray reached the level of the flue-gas exit. The powder may be transferred to a vacuum drier to complete the drying.

Previous condensation of milk or other liquid to be sprayed is a disadvantage and may cause it to be thrown onto the walls. Condensation increases viscosity and increases the size of the particles of powder resulting from the spray. These large powder grains dry superficially, but if then placed in a hermetically sealed container, the moisture from the inside of the grains appears on their surfaces. If such powder, soon after being formed, is placed in a vacuum desiccator, it may be completely dried without coalescence of the grains.

*Addenda.*—Owing to the hygroscopic nature of orange power the drying apparatus has been modified as follows: The floor of the drying chamber is made of a box 1 foot high, the top of which is made of muslin. Air dried over  $\text{CaCl}_2$  is forced into the box and comes slowly through the muslin, thus completing the drying of the powder that falls on top and keeping it cool.

## HEMATO-RESPIRATORY FUNCTIONS.

### XII. RESPIRATION AND BLOOD ALKALI DURING CARBON MON-OXIDE ASPHYXIA.

By HOWARD W. HAGGARD AND YANDELL HENDERSON.

*(Investigations performed for the United States Bureau of Mines\* in the Laboratory of Applied Physiology, Yale University, New Haven.)*

(Received for publication, May 17, 1921.)

Carbon monoxide asphyxia has long been accepted as involving the typical condition of acidosis. The decreased oxygen-carrying power of the blood was supposed to result in incomplete combustion in the tissues and a production of organic acids. The increase of lactic acid in the blood and urine (Araki (1) and Ryffel (2)) was thus explained. Without this foundation of correct observation, but incorrect inference, the theory of acidosis in a wide variety of conditions would probably never have attained the hold which it now has on current thought.

It is true, as found by Saiki and Wakayama (3), that under carbon monoxide asphyxia the blood alkali is greatly decreased. But, as we shall show, this decrease is not of acidotic origin.

In previous papers (4, 5, 6) we have demonstrated that a decrease of blood alkali may be induced in two almost diametrically opposite ways: (1) the acidotic process, and (2) the acapnial process. In the acidotic process strong acids find their way into the blood, partially neutralize the  $\text{NaHCO}_3$  of the plasma, and overload the corpuscles with acid. A differential test of this condition may be carried out by causing the subject to inhale air containing 6 or 8 per cent  $\text{CO}_2$ . If the acid intoxication is extreme, and the inhalation is pushed, the animal is soon killed by the excessive acidity thus induced in the blood. A normal animal is not harmed.

In the acapnial process, on the other hand, various influences and conditions excite the respiratory center through agencies

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other than increase of  $C_H$ . This results in overbreathing and an excessive elimination of  $CO_2$  which leaves the blood abnormally alkaline. A gradual compensatory disappearance of alkali from the blood follows. Subjects in this condition respond favorably to inhalations of  $CO_2$ . Such inhalations not only overcome the alkalosis, but also rapidly recall alkali to the blood. It will be seen in the experiments here to be reported that it is the acapnial, and not the acidotic, process which comes into play. In a condition of acidotic origin the administration of sodium bicarbonate should be beneficial. It should be injurious in acapnia; and, as we find, it is injurious and even fatal in carbon monoxide asphyxia.

All the experiments were carried out upon dogs. Enough illuminating gas, containing about 25 per cent carbon monoxide, was mixed with air in a large spirometer to afford the desired concentration of carbon monoxide, namely 15 to 45 parts in 10,000 of air, that is 0.15 to 0.45 per cent. A mask was made air-tight over the animal's head with adhesive plaster. An inspiratory valve and tube led from the spirometer to the mask, and an expiratory valve from the mask to the outside air, or at intervals to a wet gas meter of low resistance. Such conditions are quite comfortable and painless. The femoral artery was exposed under cocaine, and blood samples were taken at intervals. Part of each sample was analyzed, and part was equilibrated with three tensions of  $CO_2$  in air and analyzed, as in our previous work.

The following protocol, of which the data are given in Experiment 1 and Fig. 1, is typical. The subject breathed air containing 0.25 per cent of carbon monoxide until death resulted after 237 minutes. The volume of respiration gradually increased nearly threefold. The blood alkali, or  $CO_2$ -combining power, fell gradually, but more slowly and to a less degree than the respiration increased. Consequently, the  $CO_2$  content of the arterial blood, as the table in the protocol shows, was relatively so much reduced that the ratio  $H_2CO_3: NaHCO_3$ , and presumably therefore the  $C_H$ , was constantly subnormal. There was no acidosis, but a marked alkalosis until near the end when respiration was as usual depressed.

The most complete distinction between the acidotic and acapnial processes is to be gained by plotting the results of a series

of blood gas analyses in a  $\text{CO}_2$  diagram. Such a diagram for this experiment is shown in Fig. 1. The dissociation curves are drawn from the data of the protocol and the position of the arterial blood on each curve is indicated by a dot. The abscissæ in such a diagram are proportional to the alveolar tension of  $\text{CO}_2$  and therefore to the content of  $\text{H}_2\text{CO}_3$  in the arterial blood. The ordinates express the combined  $\text{CO}_2$  or blood alkali. The diagonal

*Experiment 1.*—Dog, female, 11 kilos. From 12 o'clock on the animal breathed air containing 0.25 per cent carbon monoxide from a large gasometer through a mask and valves. Blood drawn from the femoral artery under cocaine; part analyzed for  $\text{CO}_2$  content, remainder equilibrated with 40, 72, and 18 mm.  $\text{CO}_2$  at body temperature and analyzed.

Curve No.	Time.	Volume of respiration per minute.	Blood equilibrated with $\text{CO}_2$ .			Arterial blood.			$\text{CO}_2$ ratio.	$\text{Ca}^{+7}$ (Char. 8).
			18 mm.	40 mm.	72 mm.	$\text{CO}_2$ content.	$\text{CO}_2$ dissolved.	$\text{CO}_2$ combined.		
		liters	vol. per cent	vol. per cent	vol. per cent	vol. per cent	vol. per cent	vol. per cent		
1	11.45	4.5	23	38	48	38	2.8	35.3	0.79	0.63
	12.25	5.2	23	38	48	38	2.8	35.3	0.79	0.63
2	1.10	6.1	23	37	46	31	2.0	29.0	0.69	0.55
	1.40	6.3								
	2.00	7.4	18	32	42	25	1.7	23.3	0.73	0.58
	2.25	8.0								
	2.45	9.0	18	33	41	24	1.5	22.5	0.70	0.56
3	3.10	12.0	17	33	41	18	1.1	16.9	0.69	0.55
4	4.00	7.0	13	25	32	14	1.2	12.8	0.93	0.74

Gasps, apnea, death.

The data of this experiment are expressed graphically in Fig. 1 and are discussed in its legend.

line OC expresses the normal  $\text{C}_\text{H}$  or pH, since for every point in this line the relation of abscissa : ordinate is constant. Thus whenever the arterial point falls to the left of the OC line the ratio  $\text{H}_2\text{CO}_3 : \text{NaHCO}_3$  is below normal, and overbreathing, acapnia, and alkalosis are indicated. This condition prevailed throughout this experiment until the terminal depression of breathing set in. That these conditions are induced and determined by the volume of breathing is shown by the observations noted on the curve for the respiration (Resp.) in the figure.

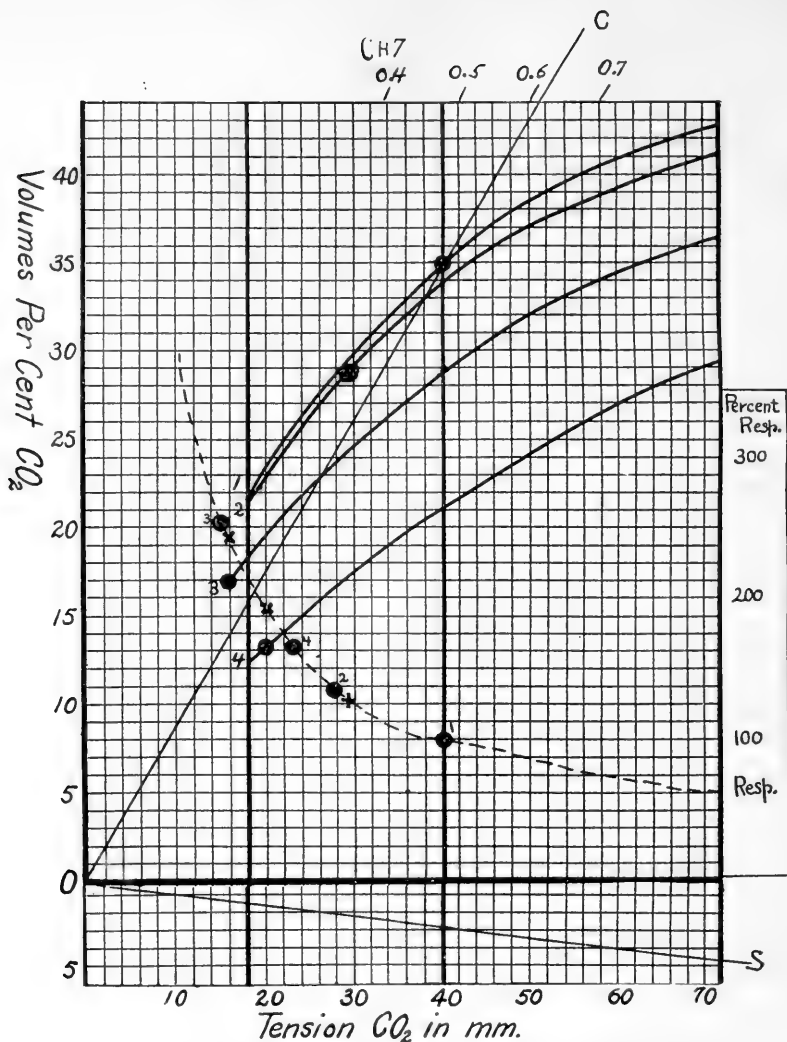


FIG. 1. The  $\text{CO}_2$  diagram (5) plotted from the data of Experiment 1. The lowering of the dissociation curve from 1 to 4, expresses the decrease of blood alkali. The line OC expresses the normal  $C_{II}$  of this animal. The deviation of the arterial points (•) to the left of the OC line on Curves 2 and 3 indicates that the excessive breathing is producing acapnia and alkalosis. Only during the terminal depression of breathing does a deviation to right, or toward acidosis, occur. The volumes of breathing (Resp.) as estimated from the arterial blood and as measured directly coincide in the first 3 sets of observations but deviate slightly from agreement in the fourth (• and × on curve marked Resp.).

The protocols of several other experiments are here given in abbreviated form. In addition to the points brought out in Experiment 1, some of these experiments throw light on additional matters. Thus in Experiments 2 and 3 the animals were removed from the gas-containing atmosphere before death, and allowed to recover. During the period of recovery respiration was for a time markedly depressed. A similar depression has been observed by us previously in experiments following true acid intoxication. It causes the ratio  $\text{H}_2\text{CO}_3$ :  $\text{NaHCO}_3$  to rise much above normal. Such an acidosis is, we think, to be regarded as a restorative process; it is an effort of the body to recall its alkali into use in the blood.

We are inclined to believe that acidosis, that is a spontaneous high ratio of  $\text{H}_2\text{CO}_3$ :  $\text{NaHCO}_3$ , or high  $\text{C}_\text{H}$ , always has this meaning. In other words alkalosis is an effort, and the normal method of the organism to lower the blood alkali and acidosis is Nature's method of calling more alkali into use (Experiments 2-5).

*Experiment 2.*—Dog, male, 16 kilos. From 12 o'clock on the animal breathed air containing 0.17 per cent carbon monoxide. At 6 o'clock it was in coma. It was then removed to fresh air, and recovered spontaneously. The blood alkali was estimated by equilibrating the blood with 40 mm.  $\text{CO}_2$  at body temperature and analyzing for  $\text{CO}_2$ . The volume of respiration is expressed in liters per minute.

	Normal.	Breathing 0.17 carbon monoxide.						Recovering.
Time.....	11.45	12.35	12.50	2.10	3.00	4.00	6.00	8.00
Respiration....	6.0	6.0	8.3	9.0	12.0	18.0	21.0	4.0
Blood alkali....	41	40	36	30	32	28	26	32
Arterial $\text{CO}_2$ ....	40	40	26	24	25	18	17	30

*Experiment 3.*—Dog, male, 11 kilos. Conditions similar to Experiment 2. Inhaled 0.16 per cent carbon monoxide from 12 to 9 o'clock. Then in coma, removed to fresh air, recovered spontaneously.

	Normal.	Breathing 0.16 carbon monoxide.						Recovering.		
Time.....	11.45	1.15	2.15	4.10	5.10	8.00	9.00	9.30	10.50	12.00
Respiration....	5.8	8.0	6.5	7.5	8.6	9.2	12.0	7.6	8.0	6.9
Blood alkali...	47	47	45	43	39	36	36	45	44	44
Arterial $\text{CO}_2$ ...	48	43	43	39	32	29	28	44	43	43

*Experiment 4.*—Dog, female, 7 kilos. Inhaled 0.49 per cent carbon monoxide from 12 o'clock on. Respiration failed at 12.40, and the heart stopped beating at 12.46.

	Normal.	Breathing 0.49 per cent carbon monoxide.		Dying.	
Time.....	11.45	12.36	12.38	12.42	12.46
Blood alkali.....	49	31	31	30	29
Arterial CO <sub>2</sub> .....	48	19	17	21	26

*Experiment 5.*—Dog, female, 7 kilos. Inhaled 0.45 per cent carbon monoxide for 44 minutes. Then 60 cc. of 2 per cent solution of sodium bicarbonate were injected intravenously. Tetany developed, respiration ceased, and death followed.

	Normal.	Breathing 0.45 per cent carbon monoxide.	Dying after NaHCO <sub>3</sub> .
Time.....	11.45	12.43	12.46
Blood alkali.....	50	38	75
Arterial CO <sub>2</sub> .....	51	20	52

Note that in these experiments the arterial CO<sub>2</sub> leads the blood alkali downward. Thus augmented breathing induces acapnia and alkalosis.

In Experiment 5 an intravenous injection of a moderate amount of NaHCO<sub>3</sub> caused death in respiratory failure and tetany. This experiment affords an instructive contrast to some other experiments recently published by one of us (7) in which it was found that when a dog remains in an atmosphere of carbon monoxide until death the presence of CO<sub>2</sub> (7 per cent) distinctly prolongs life. By tending to neutralize the alkalosis, the CO<sub>2</sub> prevents the occurrence of apnea vera, due to excessive loss of CO<sub>2</sub>, which is the common mode of death in carbon monoxide asphyxia.

#### *Oxygen Consumption and Respiratory Quotient under Carbon Monoxide Asphyxia.*

It might be supposed, if the current theory of asphyxial, or rather anoxemic, acidosis were correct, that during carbon monoxide poisoning the oxygen consumption by the body as a whole



would begin to fall (owing to lactic and other organic acids escaping oxidation) coincidentally with overbreathing. In Experiments 6 and 7 this is seen not to be the case. The respiratory quotient rises greatly, but the oxygen consumption, instead of falling, actually increases in one experiment almost to the end, due probably to the muscular exertion of the respiratory movements.

*Experiment 6.*—Dog, male, 7 kilos. The oxygen metabolism and respiratory quotient were obtained by placing the dog in an air-tight glass chamber of 300 liters capacity (minus a number of liters equal to the weight of the dog in kilos). An electric fan circulated the air in the chamber and a spray of water on the top of the chamber kept the temperature at 22°C. At intervals gas samples were drawn from the chamber and analyzed for oxygen and CO<sub>2</sub>. The decrease of oxygen (1.7 per cent per hour) and increase of CO<sub>2</sub> were sufficient for calculation of the metabolism and respiratory quotient, but insufficient to affect the condition of the animal appreciably.

After obtaining the normal figures for oxygen consumption, CO<sub>2</sub> output, and respiratory quotient, fresh air plus carbon monoxide to the desired concentration were run into the chamber and the measurements continued until death.

Time.	Remarks.	Air in chamber.		O <sub>2</sub> consumption per minute.	Respiratory quotient.
		O <sub>2</sub>	CO <sub>2</sub>		
<i>min.</i>		<i>per cent</i>	<i>per cent</i>	<i>cc.</i>	
0	Dog in chamber.	20.91	0.04		
15	Quiet.	20.50	0.41	74.5	0.80
30	"	20.08	0.73	76.0	0.76
47	Moving.	19.58	1.13	90.4	0.79
60	Quiet.	19.18	1.44	73.5	0.77

The chamber was then thoroughly ventilated with fresh air, closed again, and a charge of 1,450 cc. of pure carbon monoxide was run in.

0	Gas run in.	20.83	0.05		
15	Quiet.	20.43	0.35	72.6	0.75
30	"	19.94	0.77	89.0	0.86
39	Slightly excited.	19.37	1.31	103.5	0.95
45	Occasional gasps.	19.14	1.69	104.4	1.60
52	Frequent "	19.01	1.74	50.6	1.10
53	Respiration failed.				

*Experiment 7.*—Dog, male, 14 kilos.    Conditions as in Experiment 6.

Time.	Remarks.	Air in chamber.		O <sub>2</sub> consumption per minute.	Respiratory quotient.
		O <sub>2</sub>	CO <sub>2</sub>		
<i>min.</i>		<i>per cent</i>	<i>per cent</i>	<i>cc.</i>	
0	Dog in chamber.	20.90	0.05		
30	Dog quiet.	19.75	0.98	110	0.76
45	“ “	19.15	1.44	112	0.79

The chamber was then thoroughly ventilated with fresh air, closed again, and 1,500 cc. of pure carbon monoxide were run in.

0	Gas run in.	20.85	0.06		
30	Dog quiet.	19.64	0.96	106	0.81
45	Dyspnea.	18.94	1.56	118	0.96
62	“	18.31	2.76	102	1.90
69	Respiration failed.				

*Overbreathing and Acapnia as the Cause of Low Blood Alkali.*

It will be seen that the foregoing observations afford no support whatever to the idea that the lactic acid formed in the body during carbon monoxide asphyxia is the cause of the hyperpnea, or of the decrease of blood alkali. On the contrary the data are in complete accord with the view expressed in previous papers from this laboratory, and with the conclusions reached by Haldane and his collaborators (8) that asphyxia causes not acidosis but alkalosis. Macleod (9) suggests the view, which we had put forward (6, 10) that the lactic acid which is formed in such conditions is to be regarded, not as causing acidosis, but rather as assisting in neutralizing the alkalosis. Evidently carbon monoxide asphyxia induces overbreathing and a considerable degree of acapnia, a conclusion supported by the ill effects of injection of alkali in Experiment 5. The beneficial effects of inhalation of 6 to 10 per cent CO<sub>2</sub> in dogs after carbon monoxide asphyxia have recently been described by us (11). With Coburn (12) we have demonstrated the same beneficial effects on men in the depression following prolonged etherizations, in which condition also acapnia is a factor.

The view now receiving general assent, that asphyxia may cause overbreathing and acapnia, is decidedly at variance with

the theory that the  $C_H$  of the blood is the sole chemical factor in the control of breathing. Evidently the oxygen supply is also a factor. But merely to say that "oxygen deficiency stimulates respiration" leaves wide open the question as to how the deficiency acts. We need some substance, chemical process, or physiological mechanism to take the place heretofore erroneously assigned to lactic acid as a respiratory stimulant. It must however be a substance (if it is a substance at all) which does not act through an acid property, but as a respiratory stimulant of another order. There are such substances, but for the most part they have not been demonstrated to occur in the body. Ethyl ether in small amounts is a powerful respiratory excitant (13), and in unskillful or prolonged anesthesia may cause profound acapnia. Hydrogen sulfide, as we have found in experiments as yet unpublished, is even more potent. Indeed, inhalation of minute amounts of  $H_2S$  has in our hands proved capable of inducing the most intense hyperpnea, and subsequent fatal apnea vera. That this is not an effect of  $H_2S$  as an acid is demonstrated by the fact that intravenous injections of  $Na_2S$  are equally effective. We have recently been engaged in trying to identify in the blood some sulfur compound readily altered by oxygen, which might play the rôle of the hypothetical respiratory stimulant which we have called "respiratory X." As yet, however, our results in this search are inconclusive. The problem is clear only on one point: Oxygen deficiency does not stimulate respiration through the formation of a substance with acid properties.

*Carbon Monoxide Asphyxia after Section of the Vagi.*

It seems well established, or at least accepted, that  $CO_2$  and  $C_H$  influence respiration through action on the respiratory center itself (14). It occurred to us that perhaps oxygen deficiency, or anoxemia, stimulates not the center itself, but rather the vagus endings in the lungs. The probabilities are, of course, strongly against this hypothesis (which we are now investigating, however), and we mention it now only because it has led to an observation which is of critical importance here. Thus among other tests of this idea, we have carried out experiments on the influence

of carbon monoxide asphyxia upon respiration and blood alkali in dogs in which the vagi were cut.

The striking result of this operation (Experiments 8 and 9) is that the hyperpnea and dyspnea which are ordinarily seen under carbon monoxide asphyxia (Experiments 1 to 7) are entirely

*Experiment 8.*—Dog, male, 16 kilos. Under local anesthesia (cocaine) the trachea was exposed, cannulated, and connected with double valves, so that the expired air passed into a recording spirometer. Blood samples were obtained from the femoral artery. After the normal volume of respiration in liters per minute, and the arterial  $\text{CO}_2$  had been determined, the vagi were cut, and the minute volume of respiration was again determined. Then the inspiratory valve was connected to a large spirometer containing 0.5 per cent carbon monoxide in air, which the animal inhaled until death occurred 50 minutes later. The hemoglobin of the blood was then found to be combined with carbon monoxide to the extent of 76 per cent.

	Normal.	Vagi cut.	Breathing 0.5 per cent $\text{CO}$ .					
Time, min.....	0	15	25	35	45	55	65	75
Respiration, liters.....	3.1	3.6	3.4	3.2	3.5	3.4	2.8	0
Arterial $\text{CO}_2$ .....	48		46				48	

Note that in this and the following experiment, in marked contrast to all the preceding experiments, there was no hyperpnea and no fall of blood alkali.

*Experiment 9.*—Dog, male, 12 kilos. Conditions similar to Experiment 8. At death  $\text{HbCO} = 72$  per cent.

	Normal.	Vagi cut.		Breathing 0.5 per cent $\text{CO}$ .					
Time, min.....	0	20	25	38	45	60	70	80	
Respiration, liters.....	2.6	4.1	3.2	2.9	3.7	2.8	2.6	0	
Arterial $\text{CO}_2$ .....	40						38	Death.	

lacking after section of the vagi; and the blood alkali remains practically unaltered throughout the whole course of asphyxia, and even up to death. It is not necessary in the present connection to assign to the vagus section, or to consider here, any effect beyond the prevention of excessive breathing. This prevents the loss of  $\text{CO}_2$  (acapnia) and the usual compensatory fall

of blood alkali. If the acidotic process were involved in asphyxia the blood alkali would fall as well without hyperpnea as with it. Evidently therefore, oxygen deficiency as such does not cause a production of acid in the tissues, or at least, no passage of such acids into the blood, for the blood alkali is not at all or only slightly decreased.

This demonstration is so clear-cut a disproof of the theory of anoxemic acidosis under carbon monoxide as to bring into serious doubt the possibility of such an acidosis under any condition. The papers from this laboratory (15) a few years ago, and from other laboratories more recently in which the reasoning has been based upon the assumption that such a condition may occur, will require fundamental reconsideration. Evidently the increase of acidity in tissues to which the circulation is obstructed is wholly due to accumulation of  $\text{H}_2\text{CO}_3$  (16), and not at all to neutralization of alkali by lactic and other strong acids. When an increase of lactic acid, or rather lactate, occurs it indicates as above shown, not acidosis, but alkalosis.

#### CONCLUSIONS.

Carbon monoxide asphyxia induces, not acidosis, but alkalosis. The lowering of blood alkali is due to the acapnial, not the acidotic, process. The anoxemia induces excessive breathing (up to 300 per cent or more), and the decrease of blood alkali is an attempt, at compensation.

The rate of oxygen consumption is scarcely, if at all, decreased until death is imminent, but the respiratory quotient may be more than doubled.

After section of the vagi, on the contrary, anoxemia due to carbon monoxide causes no overbreathing, and no distinct lowering of blood alkali, even up to death. This fact, appears to be a decisive demonstration that oxygen deficiency itself does not directly cause in the tissues and blood an increased production of organic acids.

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## ANTI-KETOGENESIS.

### I. AN IN VITRO ANALOGY.\*

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(Received for publication, May 31, 1921.)

For many years the writer has been interested in the "acetone bodies," and various papers dealing with these substances have been published from this laboratory. Our main interest has been in the problems of intermediary metabolism underlying the formation of the acetone bodies; but until about 2 years ago we were not able to secure the sort of evidence which seemed to us essential for the adoption of a conception held in common with certain other workers on the subject, and our progress on these problems was slow. The missing evidence has now been found and with this encouragement the subject of ketogenesis and of the mechanism of antiketogenesis has been taken up and is being developed from several directions.

It has long been common knowledge that if any human subject fasts, or merely omits carbohydrate from his food for a few days, acetone appears in his breath, and acetone and acetoacetic and  $\beta$ -hydroxybutyric acids are excreted in his urine. The same substances are excreted in larger amounts by subjects of severe diabetes and are responsible for the acidosis and coma in this disease.

The precursors of the acetone bodies are known to be chiefly the fats and certain of the amino-acids of protein. And the fact,

\* A preliminary report of the experiments described in this paper was presented in December, 1919, before the American Society of Biological Chemists; and their application to the determination of the ketogenic antiketogenic balance in man was presented before the Federation of American Societies for Experimental Biology in December, 1920 (*J. Biol. Chem.*, 1921, xlv, p. vi).

first pointed out by Hirschfeld (1) in 1895, and since abundantly confirmed, that they appear when the amount of carbohydrate catabolized is small, demonstrates a relationship between carbohydrate metabolism and the production or the avoidance of production, of the acetone bodies. A legion of workers in the past two or three decades has tested a great variety of substances as to their effect in causing or preventing acetone body production and many of these substances have been definitely classed as either "ketogenetic" or "antiketogenetic." But efforts to explain the character of the action of antiketogenetic substances in preventing acetone body formation have not been successful and although there are many hypotheses, there is at present little direct evidence to indicate the mechanism of the reactions which may be involved.

As first suggested by Geelmuyden (2) in 1904, the most likely explanation would appear to be a definite chemical reaction between some one of the acetone bodies, or one of their precursors, and glucose, or a product of its catabolism. The detailed hypotheses of Woodyatt (3) and Ringer (4) as well as of Geelmuyden are based upon this conception, but the reactions proposed by these investigators have not been established and, perhaps in consequence, the underlying conception has not been developed and applied as it deserves. These hypotheses will be again referred to in the following paper.

With the feeling that it should be possible to find an analogy in the test-tube to the antiketogenic action of glucose in the body the writer from time to time has sought for evidence of an effect by glucose upon the oxidation of fatty acids and of the individual acetone bodies by hydrogen peroxide. The results were uniformly negative<sup>1</sup> until the combination of acetoacetic acid and glucose in alkaline solutions was tried. With this combination the results are striking. When hydrogen peroxide is added to such a mixture the acetoacetic acid disappears rather rapidly even at room temperature, the rate of disappearance increasing

<sup>1</sup> Experiments of a similar character reported by Witzemann (5) led him to the opinion (6) that the action of glucose in antiketogenesis is to "spare" butyric acid from oxidation and thus *prevent the formation of* acetone bodies. But other evidence is opposed to such a view of the nature of antiketogenesis.



with the amount of glucose and the alkalinity. In strongly alkaline solution ( $\times$  NaOH) at body temperature and in the presence of an excess of glucose and hydrogen peroxide the oxidation is complete in a few hours, while under the same conditions except for the absence of glucose, 24 hours or longer are required. In less alkaline solutions the rate of the reaction is slower, though equally striking; while in neutral or acid solution the effect of glucose is absent. With acetone, hydroxybutyric acid, or butyric acid a similar reaction has not been found. Glucose thus exhibits, in alkaline solution *in vitro*, a "ketolytic" action in hastening the oxidation of acetoacetic acid which would appear to be analogous to its "antiketogenic" action in the body. Among other antiketogenic substances so far tried, glycerol and fructose are ketolytic while lactic acid has no such action.

While the degree of alkalinity found to be necessary does not, of course, exist in the body, and one cannot therefore suppose the *in vitro* and *in vivo* reactions to be identical, there are facts suggesting that the alkalinity may concern primarily the "dissociation" or preliminary decomposition of glucose rather than other stages of the reaction, perhaps the same effect to which Woodyatt (7) has directed attention in comparing the action of alkali on sugar with the action of the pancreatic hormone. At any rate, it seems to us that the "ketolytic" action of glucose may profitably be studied on the assumption that it is similar to the reaction taking place in the body. And on the other hand, we are inclined to accept the existence of the *in vitro* reaction as strong evidence in favor of the view that antiketogenesis is to be explained by a chemical reaction between ketogenic and antiketogenic substances. In the following paper we attempt to apply this conception.

In the present paper we shall present experiments which illustrate the ketolytic action of glucose, fructose, and glycerol, and which bring out the effect of alkalinity and temperature upon the reaction. A discussion of the products of the reaction and its chemical mechanism will be postponed to a later paper.

#### EXPERIMENTAL.

Acetoacetic acid was prepared from the ethyl ester by saponification with 1 or 2  $\times$  NaOH at room temperature for 24 to 36 hours, or in the incubator over night. The solution was cooled,

acidified with sulfuric acid to Congo red paper, and extracted three to six times with a half volume of ether in a separatory funnel. The ether layer was separated and shaken in a second funnel with a small volume of water containing a few drops of alizarin red and kept alkaline by the addition of NaOH. The keto-acid is quickly and completely absorbed as sodium salt by the water, and the same ether is thus used repeatedly for extraction of the saponified ester solution. The alkaline solution of the salt is aerated by a strong air current for some hours to remove acetone, is filtered, and kept in the ice box. The acid is gradually decomposed into acetone and carbon dioxide, but in alkaline solution at room temperature or below, the rate of decomposition is slow, amounting to less than 1 per cent per day (Engfeldt (8)). When the solutions are boiled, preferably in acid solution, the keto-acid is rapidly and quantitatively decomposed into acetone which may be determined in the distillate by the iodine titration.

In alkaline solution hydrogen peroxide alone very slowly oxidizes acetoacetic acid at ordinary temperatures. At boiling temperature, during distillation the oxidation is more rapid but the rapid decomposition of the acid into acetone, which is quite resistant to peroxide and quickly distills off, prevents great loss. Oxidation during distillation may be wholly avoided by acidifying the mixture before distillation, when neither acetoacetic acid nor acetone is attacked by hydrogen peroxide, and the yield of acetone is quantitative.

The procedure usually followed in the experiments described below was to mix known amounts of sodium or calcium acetoacetate in slightly alkaline solution, sodium hydroxide, hydrogen peroxide, and glucose, some one or more of the components being omitted in various controls. All the mixtures were diluted to the same volume, usually 500 or 1,000 cc., the flasks stoppered and placed at about the same temperature. In the early experiments the solutions were at once heated to boiling and distilled. Under these circumstances the reaction takes place only until, as the temperature rises, the acetoacetic acid is decomposed into acetone and carbon dioxide.

In the later experiments the solutions were allowed to stand at room temperature or in the incubator and from time to time portions were withdrawn and the acetoacetic acid remaining,

together with small amounts of acetone formed by the slow ketone decomposition, was determined as acetone after distillation from the acid solution. As stated above the reaction does not take place in acid solution; but since volatile acids and small amounts of hydrogen peroxide may pass into the distillate from acid solutions, the first distillate was usually redistilled from alkaline solution (NaOH) or after the addition of sodium peroxide to remove possible aldehydes. In either case the peroxide is decomposed, and is without action upon acetone.

*Experiment 11.*—One of the early experiments is recorded in Table I. 50 cc. of an approximately 0.4 mol calcium acetoacetate solution were placed in each of four flasks, to which were added solutions of glucose and NaOH as stated in the table, and the mixtures were diluted to 100 cc. After 3 days at room temperature, 5 cc. portions were diluted to 300 cc. and distilled, after adding (a) 25 cc. of 5 N  $\text{H}_2\text{SO}_4$ , (b) 25 cc. of 3 per cent  $\text{H}_2\text{O}_2$  and NaOH to make the solution 0.25 N, and (c)  $\text{H}_2\text{O}_2$  and 0.5 N NaOH.

TABLE I.

*Effect of Glucose and Alkali on the Oxidation of Acetoacetic Acid by  $\text{H}_2\text{O}_2$ .*

Experiment 11. Solutions mixed and distilled after 3 days at  $20 \pm ^\circ\text{C}$ . Redistilled distillates after adding  $\text{Na}_2\text{O}_2$ .

Solution No.	In 100 cc. of solution.			First distillation from:							
	Ca acetoacetate.	Glucose.	NaOH	$\text{H}_2\text{SO}_4$	$\text{H}_2\text{O}_2 \pm$ neutral.	$\text{H}_2\text{O}_2$ in 0.25 N NaOH.	$\text{H}_2\text{O}_2$ in 0.5 N NaOH.				
Acetoacetic acid as acetone.											
Found.	Decomposed in distillation.										
	mg.	mg.	per cent	mg.	per cent	mg.	per cent	mg.	per cent		
I	20	0	0	1,114	16	1.4	138	12.5	118	10.6	
II	20	20	0	1,128	60	5.3	460	40.8	694	61.5	
III	20	0	20	1,084	72	6.6	220	20.3			
IV	20	20	20	1,064	72	6.7	492	46.2			

The results allow the following conclusions. (1) There was little, if any, disappearance of acetoacetic acid on standing at room temperature with glucose (in absence of peroxide) in either

neutral or alkaline solution. (2) On distillation in the presence of hydrogen peroxide in "neutral" solution. 1.4 to 6.7 per cent of the keto-acid was oxidized, while a much larger amount disappeared on boiling with peroxide in alkaline solution. In the absence of glucose, the loss was 10 to 20 per cent, but *in the presence of glucose 40 to 61 per cent of the acetoacetic acid disappeared*. Since the keto-acid quickly decomposes into acetone, which as other experiments show is not similarly destroyed by glucose and peroxide, *30 to 40 per cent of the keto-acid was decomposed through the presence of an equimolecular amount of glucose within the 5 or 10 minutes* taken to warm the solution to boiling and to split off acetone from those molecules of the acid which had not already been converted into a different substance. Other experiments of the same kind but with larger amounts of glucose showed almost complete decomposition of the acid.

*Experiment 14. (Table II) Distillation of Ca Acetoacetate, Glucose, and H<sub>2</sub>O<sub>2</sub>.—50 cc. of acetoacetate solution (equivalent to 0.7 millimol or 41 mg. of acetone) + 25 cc. of 5 N NaOH (= 0.4 N) + 11.5 cc. of 3 per cent H<sub>2</sub>O<sub>2</sub> (= 10 millimols) total volume 300 cc. Glucose added as below, heated slowly, and distilled 25 minutes. Distillates redistilled after acidifying with H<sub>2</sub>SO<sub>4</sub>.*

TABLE II.

## Experiment 14.

Solution No.	In 300 cc. of 0.4 N NaOH: 0.7 millimol Ca acetoacetate, 10 millimols H <sub>2</sub> O <sub>2</sub> and glucose:	Acetone found.	Acetoacetic acid oxidized.			
			Total.	Corrected for effect of H <sub>2</sub> O <sub>2</sub> alone.		
					Total.	Glucose.
	millimols	mg.	per cent	millimols	per cent	mols
I	None.	36	12			
II	0.5	18	56	0.31	43	0.6
III	1.0	5	88	0.54	76	0.5
IV	2.5	2	95	0.59	83	0.24
V	5.0	4	90	0.55	78	0.11

The results show that under the relatively unfavorable conditions during heating to boiling and distillation an excess of glucose accomplishes the decomposition of nearly all of the acetoacetic acid, and that 1 millimol of glucose is as effective (for 0.7 millimol of acetoacetate) as a larger amount.

*Experiment 18. (Table III).*—This experiment shows a marked increase in the ketolytic action of glucose with increasing alkalinity of the mixture. 25 cc. of Ca acetoacetate solution (= 0.7 millimol or 41 mg. of acetone) were added to 1 millimol of glucose and 10 millimols of  $\text{H}_2\text{O}_2$  in a volume of 250 cc., and after adding NaOH to give the final alkalinity stated, the solutions were distilled and the distillates redistilled from dilute  $\text{H}_2\text{SO}_4$ .

TABLE III.

Experiment 18. Immediate distillation.

	In 250 cc. of solution: 0.7 millimols Ca acetoacetate. 1.0 " glucose. 10.0 " $\text{H}_2\text{O}_2$ :	Alkalinity NaOH.	Acetone found.	Acetoacetate oxidized as acetone.	
				Total.	
		N	mg.	mg.	per cent
I		$\pm$ Neutral.	41		
II		0.01	36	5	12
III		0.10	28	13	32
IV		0.20	13	28	68
V		0.5	7	34	83

*Experiment 34. (Table IV) Ketolytic Action of Glucose at 20°C.*—After mixing as stated in Table IV the solutions stood in stoppered flasks at room temperature ( $20 \pm ^\circ\text{C}$ .) for 16 hours, when they were acidified with  $\text{H}_2\text{SO}_4$  and distilled. In acid solution both oxidation by peroxide and the effect of glucose are avoided, and the results represent therefore the reaction which took place at room temperature. The distillates were redistilled from  $\text{Na}_2\text{O}_2$ .

TABLE IV.

Experiment 34. 16 hours at 20°C.

Solution No.	1.5 millimols Ca acetoacetate in 250 cc. 0.2 N NaOH, containing also:	Acetone found.	Acetoacetate oxidized.
		mg.	per cent
I	Control, no $\text{H}_2\text{O}_2$ , no glucose.	73	
II	22 millimols $\text{H}_2\text{O}_2$ , no glucose.	67	8
III	22 " $\text{H}_2\text{O}_2$ + 2.5 millimols glucose.	19	74

*Experiment 44. (Table V).*—Solutions were mixed as stated below. After standing at room temperature ( $20 \pm ^\circ\text{C}$ .) for 48 hours, 50 cc. of each were acidified, diluted, and distilled. Distillates were redistilled from NaOH to remove volatile acids and peroxide.

TABLE V.

Experiment 44.

Solution No.	In 500 cc. of 0.5 N NaOH:			After 48 hours at 20°C.		
	Ca acetoacetate.	Glucose.	H <sub>2</sub> O <sub>2</sub>	Acetone found.	Acetoacetic oxidized corrected for H <sub>2</sub> O <sub>2</sub> alone.	
						For 1 mol glucose.
	millimols	millimols	millimols	mg.	mol	
I	20			1,173		
II	20		100	592	10.0	
III	20	100		958	3.7	
IV	20	4	100	360	4.0	1.0
V	20	10	100	126	6.3	0.6
VI	20	20	100	118	6.4	0.3

TABLE VI.

Experiments 45 and 54. 24 hours at 20°C.

Solution No.	Ca acetoacetate.	Glucose.	H <sub>2</sub> O <sub>2</sub>	Acetone found after 24 hrs.	Acetoacetate oxidized.		
					Total.	Corrected for H <sub>2</sub> O <sub>2</sub> .	
						Total.	Per mol glucose.
	millimols	millimols	millimols	mg.	millimols		

## 0.5 N NaOH

I	6.6	0	0	384	0		
II	6.6	0	100	259	2.15		
III	6.6	1.25	100	204	3.17	1.02	0.8
IV	6.6	2.50	100	167	3.74	1.59	0.6
V	6.6	3.75	100	125	4.46	2.31	0.6
VI	6.6	5.0	100	107	4.77	2.62	0.5
VII	6.6	10.0	100	39	5.95	3.8	0.4

## 1.0 N NaOH

I	6.0	0	35	301			
II	6.0	3.0	35	142		2.74	0.91
III	6.0	5.0	35	117		3.18	0.63

From the results of this experiment it appears that if allowance be made for the amount of acetoacetic acid oxidized by peroxide in the absence of glucose, glucose may accomplish the transfor-

mation of an equimolecular amount of acetoacetic acid when reacting upon a large excess of the keto-acid (Solution IV). In the mixtures with a larger relative amount of glucose, its apparent effect is less, due in part to the error in assuming the full effect of peroxide alone in these solutions. It may be noted also that a large excess of glucose, even without peroxide (Solution III) caused some disappearance of keto-acid. This has been occasionally noted at high alkalinity, but has not been further investigated.

*Experiments 45 and 54. (Table VI).*—The procedure was the same as in Experiment 44. The solutions stood 24 hours at room temperature ( $20\pm^{\circ}\text{C}$ ).

*Experiment 56.*—After mixing the solutions as stated in Table VII, they stood at  $30\pm^{\circ}\text{C}$ . for 20 hours, after which portions of each were distilled from sulfuric acid, and the distillates redistilled from NaOH.

TABLE VII.

Experiment 56.

Solution No.	Ca acetoacetate.	Glucose.	$\text{H}_2\text{O}_2$	Acetone found.	Acetoacetic oxidized in 20 hrs.		
					Total.	Corrected for $\text{H}_2\text{O}_2$ .	
							Per mol glucose.
	millimols	millimols	millimols	mg.	millimols	millimols	
In 500 cc. of 0.25 N NaOH:							
I	11	0	0	668			
II	11	0	100	412	4.42		
III	11	11	100	78	10.15	5.72	0.52
In 500 cc. of 0.5 N NaOH:							
IV	11	0	0	632			
V	11	0	100	366	4.5		
VI	11	11	100	20	10.5	6.0	0.54

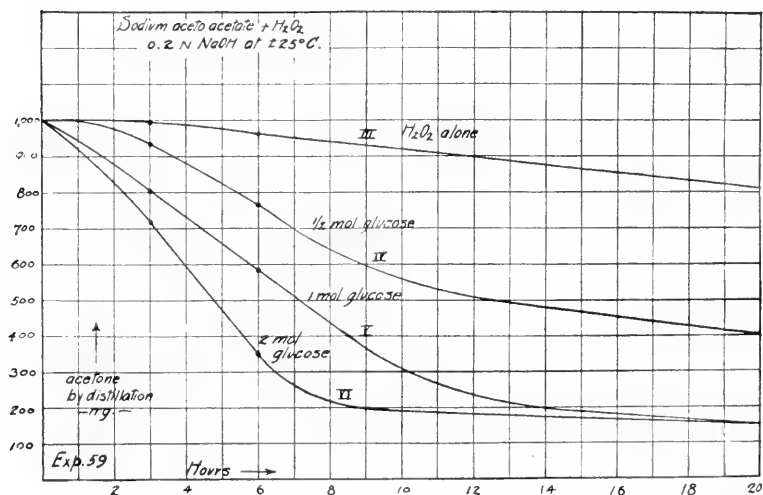
*Experiment 59. (Table VIII).*—The results of this experiment are plotted in curves in Fig. 1, which shows graphically the relative rate of the decomposition of acetoacetic acid under these conditions, in the presence and absence of glucose.

*Experiment 65. (Table IX).*—Rate of reaction at  $30\pm^{\circ}\text{C}$ . The details are given in Table IX and are shown in the form of curves in Figs. 2 and 3. Attention may be called to the following points: (a) the slow oxidation of acetoacetic acid in the form of its sodium salt in 0.25 N NaOH by  $\text{H}_2\text{O}_2$

TABLE VIII.

Experiment 59.

Solution No.	Na acetoacetate + glucose + $H_2O_2$ at $25 \pm ^\circ C$ .						
	In 1,000 cc. of 0.2 N NaOH:			Acetone by distillation.			
	Na acetoacetate.	Glucose.	$H_2O_2$	Start.	3 hrs.	6 hrs.	22 hrs.
	millimols	millimols	millimols				
I	16.8	0	0	968	932	912	956
II	16.8	16.5	0	944	930	908	892
III	16.8	0	100	1,024	942	962	810
IV	16.8	8.2	100	1,068	936	772	386
V	16.8	16.5	100	996	804	588	140
VI	16.8	33	100	1,036	722	352	148

FIG. 1. Experiment 59, showing rate of ketolytic action in 0.2 N NaOH at  $25 \pm ^\circ C$ , by 0.5, 1.0, and 2.0 molecular equivalents of glucose.

at  $30^\circ C$ .; (b) the increasing speed of its disappearance with increasing amounts of added glucose and with increasing alkalinity (Fig. 2); and (c) the fact that an equimolecular amount of glucose (Solution V) was required to accomplish almost complete decomposition, indicating a 1:1 molecular relationship between glucose and acetoacetic acid in the reaction. The slope of the curve with one-half molecular equivalent of glucose is slightly greater than expected and is perhaps due to error in the



determination, or the glucose solution added was possibly slightly stronger than was supposed.

Fig. 3 shows the marked increase in the speed of the reaction with increasing alkalinity (Solutions III, V, X, and XI).

TABLE IX.

Experiment 65. Sodium acetoacetate and glucose at  $30 \pm ^\circ\text{C}$ . At end of period solutions were acidified and distilled. Redistilled from NaOH.

Solution No.	In 1,000 cc. 0.25 N NaOH:			Acetone recovered by distillation, representing the unoxidized acetoacetate after:						
	Na acetoacetate.	Glucose.	H <sub>2</sub> O <sub>2</sub>	12 min.	25 min.	60 min.	125 min.	235 min.	420 min.	24 hrs.
	milli-mols	milli-mols	milli-mols							
I	20	0	0		1,204		1,206	1,208		1,148
II	20	20	0		1,204		1,196	1,196		1,148
III	20	0	150		1,212		1,198	1,172	1,120	1,020
IV	20	10	150		1,216		1,164	1,054	812	244?
V	20	20	150		1,198		1,128	916	524	32
VI	20	40	150		1,112		1,004	696	182	38
VII	20	20D <sub>1</sub>	150		1,118		844	612	352	39
					30 min.		150 min.		300 min.	
VIII	20	20D <sub>2</sub>	300	1,180	1,112	1,088	1,018		942	
IX	20	10D <sub>2</sub>	300	1,196	1,154	1,128	1,052		976	
In 0.5 N NaOH:						80 min.		260 min.	375 min.	
X	20	20	150	1,202		1,096		488	128	26
In N NaOH.										
XI	20	20	150	1,204		1,068		138	56	26

Another point of great interest is shown in this experiment. It will be seen that in Mixtures IV, V, and VI (Experiment 65, and Fig. 2) in which neutral, fresh solutions of glucose were added simultaneous with the peroxide, there was an interval of an hour or less before the decomposition or disappearance of acetoacetic acid began, after which, as shown by the shape of the curves, the rate of the reaction increased to a maximum. If, however, the glucose is first allowed to stand for some hours with alkali, during which time various rearrangements and "dissociations"

of glucose take place, as shown by the well known work of van Eckenstein, de Bruyn, and Nef (9), the reaction with acetoacetic acid and peroxide begins at once when the last two are added. In Mixtures VII, VIII, and IX of Experiment 65 the glucose used had been previously "dissociated" by standing at room temperature for 16 hours in about 0.3 N NaOH ( $D_1$  in Solution

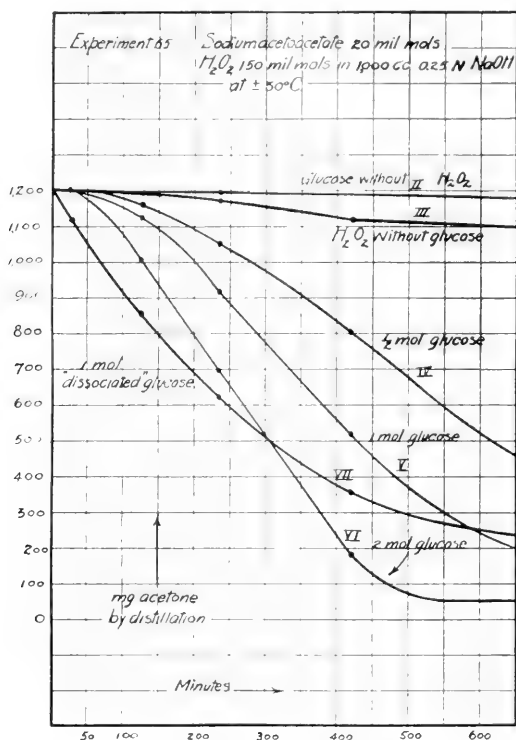


FIG. 2. Experiment 65, showing rate of ketolytic action of ordinary glucose as compared with the faster rate with glucose previously "dissociated" by treatment with alkali.

VII) and 0.6 N NaOH ( $D_2$  in Solutions VIII and IX). For at least 5 hours the rate of reaction of Solution VIII exceeded the rate with double the amount of ordinary glucose, Solution VI, after which time the rate falls off, but continues to exceed that of Solution V containing the same amount of "undissociated" glucose. The same phenomenon is observed in Solutions VIII

and IX but in these cases the initial rate more quickly falls off, perhaps due to an injurious effect of the stronger alkali (0.6 N) on the glucose. The results in these experiments are less striking and their curves are omitted from the figure. Similar experiments have been repeated a number of times with essentially the same result. The rate of the oxidation of glucose alone, by peroxide, is likewise affected by previous treatment with alkali, and there appears to be no doubt that the rate of "dissociation" by alkali is one of the limiting factors determining the rate of the reaction with acetoacetic acid.

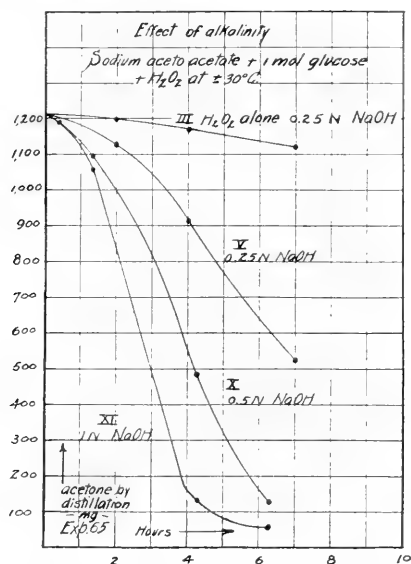


FIG. 3. Experiment 65, showing increasing rate of ketolytic action with increasing alkalinity.

It is evident from this behavior that glucose must be either first "dissociated" or transformed into some other more active substance before it takes part in this reaction. This fact is at least suggestive when it is recalled that glucose is not antiketogenic in the severe diabetic who is unable to metabolize it. One is tempted to wonder whether the diabetic could use glucose after it had been "dissociated" for him by appropriate treatment with alkali. Further consideration of this matter will be reserved for a later paper.

*Ketolytic Action of Glycerol, Fructose, and Lactic Acid.*

The results given below in Tables X, XI, and XII, and in Figs. 4 and 5, representing Experiments 63, 68, and 69 show that fructose and glycerol have marked ketolytic action, similar to and perhaps even exceeding that of glucose. Both are antiketogenic when fed to diabetics. Lactic acid on the other hand appears not to be capable of affecting the decomposition of acetoacetic acid under the conditions of the *in vitro* experiments. Further experiments with these and other substances are in progress and will be reported in a later paper.

TABLE X.

Experiment 63. *Na Acetoacetate + Fructose + H<sub>2</sub>O<sub>2</sub> at 30°C.*

Solution No.	In 250 cc. 0.5 N NaOH:			Acetone by distillation from H <sub>2</sub> SO <sub>4</sub> and redistillation from NaOH.		
	Na acetoacetate.	Fructose.	H <sub>2</sub> O <sub>2</sub>	Total for 250 cc.		
				Start.	1½ hrs.	3¼ hrs.
	<i>millimols</i>	<i>millimols</i>	<i>millimols</i>			
I	5	5	100	238	61	39
II	5	0	100	235	226	214
III	0	5	100	11		

TABLE XI.

Experiment 68. *Ketolytic Action of Glycerol and Lactic Acid at 38°C.*

Solution No.	In 1,000 cc. normal NaOH: 20 cc. mol Na acetoacetate. 200 " " H <sub>2</sub> O <sub>2</sub> and:	Acetone by distillation from H <sub>2</sub> SO <sub>4</sub> and redistillation from NaOH.		
		3¼ hrs.	5½ hrs.	23½ hrs.
I	Control, H <sub>2</sub> O <sub>2</sub> alone.	(1,090)	(1,030)	(550)
II	20 mol glucose.	221	82	93
III	20 " glycerol.	535	148	54
IV	20 " lactic acid.	985	834	442

TABLE XII.

Experiment 69. *Ketolytic Action of Lactic Acid at 38°C.*

Solution No.	In 1,000 cc. of normal NaOH: 20 cc. M Na acetoacetate + 200 cc. M H <sub>2</sub> O <sub>2</sub> , and:	Acetone by distillation from H <sub>2</sub> SO <sub>4</sub> and redistillation from NaOH.			
		1 hr.	2 hrs.	3¼ hrs.	19 hrs.
	Control, H <sub>2</sub> O <sub>2</sub> alone.	1,122		982	547
	20 M lactic acid.	1,148	1,117	1,068	640

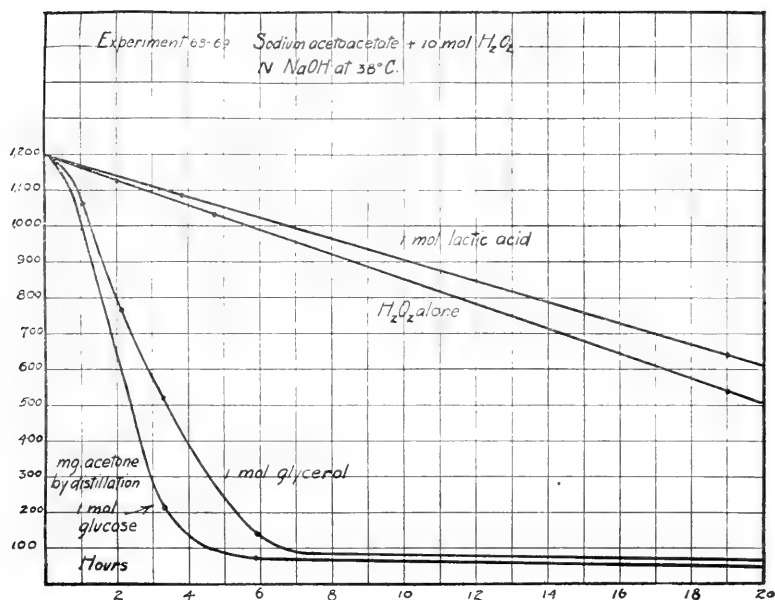


FIG. 4. Experiments 68 and 69, showing rate of ketolytic action of glucose and glycerol, and absence of such action by lactic acid, in normal NaOH at  $38 \pm^\circ C$ .

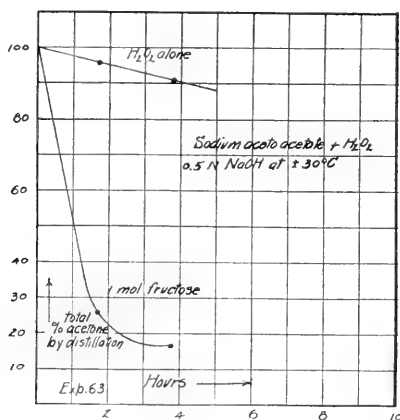


FIG. 5. Experiment 63, showing rapid ketolytic action by fructose in 0.5 N NaOH at  $30 \pm^\circ C$ .

## SUMMARY.

The foregoing experiments demonstrate that the oxidation of glucose in alkaline solution by hydrogen peroxide accomplishes the disappearance of acetoacetic acid if the latter be present in the solution. Acetoacetic acid in the absence of glucose or other "ketolytic" substance is oxidized very slowly by hydrogen peroxide, but its disappearance is rapid even at room temperature if glucose is simultaneously oxidized. Fructose and glycerol exert the same effect as glucose, while lactic acid is without such action.

The rate of the "ketolytic action" is increased with alkalinity, temperature, and amount of glucose or other ketolytic substance. The rate of the reaction appears to be determined primarily by the rate of the "dissociation" or the conversion of glucose by alkali into a derivative which is then oxidized. The inference seems justified that it is some intermediate oxidation product of glucose which combines with acetoacetic acid, the compound being then further oxidized. The details of the reaction and its products will be considered in a separate paper.

The phenomenon is believed to be an *in vitro* analogy to the action of glucose and of similar substances in abolishing or preventing the formation (accumulation) of acetoacetic acid and the related acetone and  $\beta$ -hydroxybutyric acid in man.

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## ANTI-KETOGENESIS.

### II. THE KETOGENIC ANTI-KETOGENIC BALANCE IN MAN.

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(Received for publication, May 31, 1921.)

In the preceding paper (1) it has been shown that glucose and related substances such as fructose and glycerol exert a marked effect upon the oxidation of acetoacetic acid by hydrogen peroxide in alkaline solution. At room temperature or in the incubator, and in the absence of glucose, alkali salts of acetoacetic acid are only very slowly oxidized by hydrogen peroxide; but if glucose is present the disappearance of the keto-acid is rapid, the rate increasing with the temperature, with increasing alkalinity of the solution, and with increasing amounts of glucose. Although the details of the reactions are not yet fully known, it is evident that a chemical reaction occurs between some derivative of glucose and acetoacetic acid, involving definite molecular quantities of each substance. It is likewise evident that this "ketolytic" action of glucose *in vitro* is strikingly similar to the long known "antiketogenic" action of glucose and related substances in preventing the appearance of the acetone bodies in man. Although the degree of alkalinity used in the experiments is not approached in the body, the cells probably possess, as pointed out by Woodyatt (2), some means of producing a similar effect. As explained in the preceding paper, it was the search for a chemical explanation of "antiketogenesis" which brought to light the behavior of glucose there described.

The fact that the ketolytic action of glucose *in vitro* is a chemical reaction suggests that the effect of carbohydrate in preventing or abolishing ketonemia in man is also the result of a definite chemical reaction in the tissues.

The latter conception is by no means new as will be shown by the review of the literature, but the various hypotheses in which

such conceptions have been advanced have not been established, and there is at present no accepted explanation of the mechanism of antiketogenesis.

The literature on the subject under discussion is so large that its detailed and complete review will not be attempted, but brief reference to a few of the more important investigations and hypotheses concerning antiketogenic action is necessary.

The relationship between acetonuria and carbohydrate starvation, first pointed out by Hirschfeld (3) and Rosenfeld (4) has been abundantly established. All of the various conditions in which acetone and the related two acids are excreted appear to have in common the fact that abnormally small amounts of carbohydrate are being *burned*. The formation of acetoacetic and  $\beta$ -hydroxybutyric acids does not represent a qualitative disturbance of metabolism; since both are readily oxidized when given to man and animals (5-7), it is more reasonable to believe that they represent normal intermediates in metabolism, which, in the absence of sufficient carbohydrate combustion, escape further oxidation (Geelmuyden, 8). When they accumulate in the tissues, acetone, which is not a normal intermediate, is formed by decomposition of acetoacetic acid (8). Geelmuyden (8) in 1904 first advanced the hypothesis that carbohydrate and acetone bodies react in their intermediary metabolism by a synthesis which is necessary for the further decomposition of the acetone bodies; and that failing this synthesis their accumulation and excretion result. He assumed a conjugation with glycuronic acid as explaining the character of the reaction, and called it "die Hypothese von der chemischen Interferenz der intermediären Stoffwechselproducte." In a later paper, Geelmuyden (8) reiterates this view, but no experimental evidence was adduced as to the qualitative or quantitative aspects of the chemical reactions between the acetone bodies and the derivative of glucose. Rosenfeld (9) proposed the less definite conception that carbohydrate serves to catalize or set off the oxidation of fats, "Der Zündstoff für die Fette sind die Kohlenhydrate."

That such ideas failed to take root is indicated by the remark of von Noorden (10), who, in his Handbuch concludes the discussion of the action of carbohydrate in preventing acetone body formation with the statement, "Wie die Kohlenhydrate und antiketogenen Substanzen es bewirken . . . wissen wir nicht."



In 1910 Woodyatt (11) clearly formulated the problem and set forth a speculative explanation on the assumption that "antiketogenesis is an effect due to certain products which occur in the oxidation of glucose, an interaction between these products on the one hand and one or more of the acetone bodies on the other." The conception advanced by Woodyatt was suggested by the work of Ciamician and Silber on the reciprocal oxidation and reduction of alcohols and ketones, keto-acids and other substances when exposed to sunlight, and was to the effect that acetoacetic acid, the first formed of the acetone bodies, undergoes a reciprocal reduction by the simultaneous oxidation of glucose, glyceric aldehyde, or other antiketogenic substance. While Woodyatt's assumption above quoted and as later elaborated (12) appears to be supported by the results in the preceding paper, no direct evidence has been advanced concerning the mechanism of the reactions which he proposed.

A third "chemical interference hypothesis" was presented in 1914 by Ringer (13) who conceived "the rôle of glucose in the normal individual in preventing acidosis to be such as to deviate the  $\beta$ -hydroxybutyric acid from its ordinary course of oxidation, by combining with it and thereby changing its structural configuration so as to give rise to non-acetone genetic products." Structural formulas were given of various hypothetical reactions, the experimental basis being the earlier finding by Ringer and Frankel (14) that acetaldehyde and propyl aldehyde when given subcutaneously to diabetic dogs produced a fall in the acetone body excretion and a rise in the sugar excretion. Ringer and Frankel concluded that all aldehydes including glucose react with  $\beta$ -hydroxybutyric acid and that the metabolism of the resulting compounds results not only in the disappearance of  $\beta$ -hydroxybutyric acid but also in the conversion of the latter, in part, into glucose, thus in effect accomplishing the formation of glucose from a derivative of fatty acid.

Later work by Sansum and Woodyatt (15), however, appears to show that the "extra" glucose found in Ringer and Frankel's experiments is due to the sweeping out of sugar from stored glycogen as a result of the narcotic effect of the aldehyde, and thus throws doubt upon Ringer's interpretation. Sansum and Woodyatt conclude that "the hypotheses of antiketogenesis and diabetes

which are based on the assumption that acetaldehyde promotes a new formation of sugar from fat are wholly untenable."

In view of the fact that ketonuria in non-diabetic subjects results from a deficient supply of carbohydrate food, and that in the diabetic it is associated with an inability to metabolize carbohydrate, it has long been appreciated that the cause for the formation of the acetone bodies is in both essentially the same. This point of view has been well emphasized by Woodyatt (12); and its acceptance would seem to be necessary if antiketogenesis is of the nature of a chemical reaction between definite molecular quantities of ketogenic and antiketogenic substances. But as a result of experience with the fasting treatment for diabetes advocated by Allen, the view is now expressed that an essential difference does exist between the ketonuria of diabetic and non-diabetic subjects. Joslin (16), for instance, writes:

"The anomaly becomes greater when it is considered that a healthy individual, fasting or on a non-protein [(?) non-carbohydrate] diet, develops an acidosis, whereas a severe diabetic treated exactly in the same manner loses an acidosis. There is surely something here which is not understood. It would appear that the diabetic individual is able to manufacture and utilize some substance which in its combustion is able to prevent acidosis, but yet a material which is not available to the normal individual."

Furthermore, the view is held that not only do different subjects show different reactions to fasting or carbohydrate starvation, but that an adaptation from training may render a subject more or less immune to the deprivation of carbohydrate. Thus Joslin, under the heading "adaptation of the body to a non-carbohydrate diet and to acidosis" states:

"It is reasonable to conclude that the body of the fasting man adapted itself to the changed conditions and in some way restricted the output of acid bodies. This may be the explanation of the lack of acidosis among the Eskimos. This power of adaption is certainly an important factor.  
. . . . ."

Folin and Denis (17) similarly interpret their observations on the ketonuria of two obese women during successive fasts. They state that their results "suggest with regard to the complete oxidation of body fat in starvation that the human or-

ganism is capable of at least a certain amount of adaptation and that it is this individual factor rather than the tendency to obesity or the extent of the fat deposits in the body which chiefly determines the onset and the degree of acidosis." And they "therefore concluded that one of the effects of repeated fastings is habituation to the complete oxidation of mobilized body fat, and a consequent retardation of the development of acidosis."

As representing still a different view the statement of von Fürth may be cited. He (18) writes:

" . . . . the distinct 'antiketogenic' influence of carbohydrates . . . . may be fully explained on the basis of an inhibition of fat decomposition because of the combustible material thus introduced into the body. . . . this makes all other complicated theories entirely superfluous, especially such theories as that of Geelmuyden. . . . "

If von Fürth means that carbohydrate acts merely by sparing from oxidation an isodynamic amount of fat, his view is incompatible with the fact that the effect of carbohydrate in preventing ketonuria is much greater than its relative caloric value.

It will be evident from the above review that antiketogenesis still awaits a satisfactory explanation. In the hope of being able to obtain new evidence, and stimulated by the results of the *in vitro* experiments described in the preceding paper, the writer has undertaken to analyze certain data from the literature and to conduct experiments of his own for the purpose of answering the question: *Is there a definite and constant mixture of foodstuffs, corresponding to definite molecular proportions of the precursors of the "acetone bodies" on the one hand, and of glucose or related substances on the other, which just suffices to avoid the appearance of the "acetone bodies" in different subjects?* If the existence of such molecular proportions could be established, it would be a convincing argument in favor of the essential similarity of the ketolytic action *in vitro* to the reaction in the body and would doubtless make possible the study and explanation of many factors in the intermediary metabolism of fats and carbohydrates which are now obscure. For if antiketogenesis is the result of a definite chemical reaction between *definite* and *constant* proportions of the reacting substances we should expect different human subjects, whether the normal, the Eskimo, or the diabetic,

to differ as to the formation of acetone bodies only as far as the relative amounts of ketogenic and antiketogenic substances metabolized are different.

The writer believes that acetone body production by all human subjects can be harmonized with this point of view, which is essentially the view proposed by Woodyatt (12) in 1916, but not so far established.

Two methods appear to be suitable for the investigation of the question above stated. From the data of respiration experiments it is customary to calculate, by means of the non-protein respiratory quotient, the amounts of fat and carbohydrate as well as of protein being catabolized. By a simple modification of the calculation one may learn the molecular proportions of the three foodstuffs; and from these data one may attempt to calculate the molecular equivalents of precursors of acetone bodies and of the substances which prevent their appearance. In this way it may be possible to learn whether all subjects who are excreting traces of the "acetone bodies" and are, therefore, on the border-line of ketogenesis and of ketonemia, are catabolizing in their bodies as a whole the same molecular proportion of ketogenic and antiketogenic substances, which proportion, if constant, will evidently be near the minimum ratio required to avoid the appearance of "acetone bodies." Methods of calculating such information and the results of their application to the problem will be presented in later papers of this series.

A second method of obtaining the same information is to feed to various subjects diets in which the proportion of protein, fat, and carbohydrate is varied and to calculate the molecular proportions of mixtures which cause the production and excretion of border-line traces of the "acetone bodies."

It is proposed in the present paper to analyze certain data of this kind, taken chiefly from the published work of others. Other similar data of our own experiments will be presented at a later time. Investigations by the same general method are being conducted also in other laboratories as evidenced by the recent papers of Palmer (19) and Woodyatt (20).

✓ If the mechanism of antiketogenesis in the body may be looked upon as similar to the action of glucose in accomplishing the further decomposition of acetoacetic acid *in vitro*, one may suppose

that acetoacetic acid is constantly formed in the body from the fatty acids and other ketogenic substances and that if the derivative of glucose and other antiketogenic substances is locally present it reacts with the keto-acid, and the product is further oxidized.

Whenever the rate of formation of acetoacetic acid exceeds the rate of formation of the antiketogenic substance, the excess of the former fails to take its usual course and as it accumulates is in part decomposed into acetone and reduced to  $\beta$ -hydroxybutyric acid, all of which substances are excreted. Acetone appears to be oxidized slowly if at all in the body, whether acetoacetic and hydroxybutyric acids are oxidized without the intervention of antiketogenic (ketolytic) substance cannot be decided at present.

The problem then is to calculate the total molecular amounts of ketogenic and of antiketogenic substances in those diets, the metabolism of which just causes the definite appearance of small amounts of the acetone bodies.

Although existing information scarcely permits an exact calculation, we may make certain assumptions and learn from their application how far the calculations based upon them are in accord with the facts. The assumptions which appear to be justifiable for a first trial, are indicated below. They are of course subject to modification as further facts are established.

*Ketogenic.*—The main source of acetoacetic acid is the fatty acids, which are assumed to form an equimolecular amount of the keto-acid. Based on this assumption Magnus-Levy (21) calculated a possible production of 36 gm. of hydroxybutyric acid from 100 gm. of fat. Taking 874 as the molecular weight of the mixed body fat one may calculate that 1 gm. of such mixed fat can give rise to  $\frac{1}{874} \times 3 = 0.00343$  gm. molecule of ketogenic fatty acid  $\times 102 = 0.35$  gm. of acetoacetic acid.

Another source of ketogenic substances is protein, since leucine, phenylalanine, and tyrosine have been found to be convertible into the acetone bodies when fed to diabetics (22), and when added to blood in liver perfusion experiments (23). Assuming that these observations indicate the normal path of catabolism for these amino-acids, the ketogenic value of protein may be calculated from the analysis of ox muscle by Osborne after the method

used by Lusk (24) in calculating the theoretical derivation of glucose. The data are given in Table I and show that each gm. of nitrogen is equivalent to approximately 0.010 gm. molecule of ketogenic substance. In the same table are given also the equivalent amounts of the four amino-acids, valine, lysine, histidine, and tryptophane, which are apparently neutral as to ketogenesis.

*Antiketogenic.*—Carbohydrates are, par excellence, antiketogenic. Until the active derivative of glucose is known we may

TABLE I.

*Amino-Acids in 100 Gm. of Muscle Protein (Osborne's Analysis as Modified by Lusk\*).*

		Molecular weight.		Remarks.
	gm.		gm. mole- cule	
Leucine.....	14.3	131	0.109	Form acetoacetic acid.
Phenylalanine.....	4.5	165	0.027	
Tyrosine.....	4.4	181	0.024	
Sum for 16.18 gm. of nitrogen .....			0.160	Ketogenic.
For each gm. of nitrogen .....			0.010	
Valine.....	2.0	117	0.017	Probably neutral as to ketogenesis.
Lysine.....	7.6	146	0.052	
Histidine.....	4.5	155	0.029	
Tryptophane.....	(2.0)?	204	0.01	
Sum for 16.18 gm. of nitrogen .....			0.099	

\* Lusk, G., Science of nutrition, Philadelphia, 3rd edition, 1917, 77.

assume that one molecule of monosaccharide is equivalent to one molecule of the active derivative, and on this basis calculate the antiketogenic value of carbohydrate in terms of molecules of glucose. Each gm. of glucose would thus be equivalent to

$\frac{1}{180} = 0.00556$  gm. molecule; and each gm. of starch to

$\frac{1}{162} = 0.00618$  gm. molecule.

Protein is known to be converted into glucose by the diabetic organism to the extent of approximately 3.6 gm. for each gm.

of nitrogen; and there is no reason to suppose that the sugar-forming amino-acids do not pass through the same stage, glucose or its derivative, in the normal individual as well. 1 gm. of urine nitrogen would then correspond to  $\frac{3.6}{180} = 0.020$  gm. molecule of glucose.

A third probable source of antiketogenic substance is the glycerol of fat, though the evidence is perhaps not conclusive. When given as such, glycerol is converted into glucose by the diabetic (25), and is antiketogenic (3, 26, 29), but its fate when ingested in the form of glyceride has not been determined. That an increase in the fat metabolized by diabetics appears not to cause an increased excretion of glucose, may indicate that as glyceride it behaves differently. But in view of the definite conversion to glucose when fed as such, its antiketogenic action, and its chemical relationship it seems preferable for the present to include the glycerol of fat among the antiketogenic substances by supposing that in its catabolism it passes through the stage of glucose or its derivative, in which form it is available for reaction with acetoacetic acid.

Its antiketogenic value, calculated in terms of glucose, is as follows.

1 gm. of fat =  $\frac{1}{874} = 0.00114$  gm. molecule fat  $\div 2 = 0.00057$  gm. molecule of glucose from glycerol.

The above calculations are summarized below.

Ketogenic substance expressed as gm. molecule of precursors of acetoacetic acid.

$$(a) \text{ 1 gm. of fat} = \frac{3 \times 1}{874} = 0.00343 \text{ mol.}$$

$$(b) \text{ 1 gm. of urine nitrogen} = 0.010 \text{ mol.}$$

Antiketogenic substance expressed as gm.-molecular equivalents of glucose.

$$(c) \text{ 1 gm. of urine nitrogen} = \frac{3.6}{180} = 0.020 \text{ mol.}$$

$$(d) \text{ 1 gm. of glucose from carbohydrate} = \frac{1}{180} = 0.00556 \text{ mol.}$$

$$(e) \text{ 1 gm. of fat} = \frac{1}{874} \div 2 = 0.00057 \text{ mol.}$$

The sum of the values of (*a*) and (*b*) divided by the sum of the values of (*c*), (*d*), and (*e*) gives the ratio of ketogenic to antiketogenic substance for the mixture metabolized, and if the general conception is correct, this ratio should determine whether or not the subject will form and excrete acetone bodies.

Before applying this calculation to observed data it may be well to state that it is of course the mixture *metabolized* rather than the mixture of foodstuffs eaten which must be considered. It is therefore necessary to determine or reasonably assume the material being *burned*. Also, it is realized that the mixture metabolized is perhaps different in different cells or different parts of the body at any one time, and that the mixture burned in the body as a whole is constantly changing. But the blood carries the same mixture of foodstuffs to all parts of the body and although the mixture varies from time to time the single source of supply probably tends to maintain an approximately uniform mixture of fuel for all cells at a given time.

It must also be pointed out that the relative values given in some of the above calculations are admittedly doubtful. The results to be presented seem to indicate (see p. 469) that the ketogenic factors of fat and of protein are correctly estimated, but the values assigned to the antiketogenic factors will probably need revision. It is quite possible for instance that the two carbon residues from glycolic and the three carbon residues from other sugar-forming amino-acids may have direct and immediate antiketogenic (ketolytic) action without condensation to glucose, and the same may be true of glycerol. While these and other uncertainties are fundamental and must be decided, they should, I believe, not deter us from the better understanding of the nature of antiketogenesis and the consequent aid in dietetics which the application of such a conception appears to make possible.

*Data of Zeller (27).*—In a study of the effect of low carbohydrate diets upon protein metabolism, Zeller records the observation that acetone did not appear until the carbohydrate was reduced to 10 per cent, and the fat increased to 90 per cent of the total calories of food supplied (3,200 calories). This diet contained 310 gm. of fat and 80 gm. of carbohydrate, and Zeller concluded that one part of carbohydrate is required for complete combustion of four parts of fat.



Lusk (28) calculates from Zeller's data that perhaps each molecule of hydroxybutyric acid requires the presence of a triose molecule; and Woodyatt (12), citing the same data, concludes that "when the mixture of metabolites oxidizing in the body, contains more than three molecules of higher fatty acids to one of glucose, then the body 'smokes' with acidosis compounds like an automobile smokes with too much oil in the cylinders."

I have recalculated Zeller's data for the diet which first produced acetonuria, as follows. The subject's total metabolism may be approximately calculated from his body weight, 80 kilos, height 175 cm. = 1.95 sq. m. (Du Bois).

	<i>calories</i>
$1.95 \times 40 \times 24$ hours = basal metabolism.....	1,872
10 per cent for effect of food.....	187
10 per cent for 8 hour's rest.....	62
50 per cent for 8 hour's work in the laboratory.....	310
Estimated total energy exchange.....	2,431

In view of the previous low carbohydrate diet it is assumed that all of the ingested carbohydrate was burned and that the fat burned is represented by the difference between the total calories and the sum of the calories from protein and carbohydrate.

		<i>calories</i>
Protein (3.67 gm. $\times$ 26.5 calories)	=	97
Carbohydrate (80 $\times$ 4.1)	=	328
Total.....		425

$2,431 - 425 = 2,006$  calories from fat  $\div 9.3 = 216$  gm. of fat burned. The ketogenic and antiketogenic balance may then be calculated.

	Ketogenic.	Antiketo- genic.	Ratio.
80 gm. of carbohydrate.....		0.445	
Protein, 3.67 gm. of N.....	0.0367	0.0634	
216 gm. of fat.....	0.741	0.123	
Total.....	0.777	0.631	1.23:1

From these results it is evident that this subject first excreted acetone when the molecular ratio of ketogenic to antiketogenic

substances burned (as above calculated) somewhat exceeded 1. With a smaller amount of carbohydrate food Zeller states that more acetone was excreted, but he gave no data as to the amount.

*Experiments of Lang (29).*—Lang reports a series of experiments upon himself, in which he determined the amount of acetone bodies excreted while on different low carbohydrate diets and during fasting. In the discussion he<sup>1</sup> states:

“When this ratio (of fat to carbohydrate oxidized) rises above a certain definite value, abnormal quantities of acetone bodies appear in the urine. The results . . . point to the existence of a quantitative relationship, which has a very definite minimum value, between the fat and the carbohydrate utilized to prevent acidosis.

“These substances (acetone bodies) appear in abnormal amounts immediately the ratio of fat to the carbohydrate burnt becomes greater than about 2 to 1.” The ratio refers to grams.

I have calculated the molecular ketogenic balance in some of his experiments, of which the following is the best example. He reports the data given below for periods during which the diet was only 60 and 100 gm. of cane-sugar daily.

Day.	Diet.	Total acetone and acetoacetic acid.	Hydroxybutyric acid.	Total nitrogen.
		<i>mg.</i>	<i>mg.</i>	<i>gm.</i>
1	60 gm. sucrose.	21	186	8.5
2	60 “ “	45	224	8.0
3	60 “ “	79	214	9.7
4	100 “ “	6.6		6.9
5	100 “ “	9		11.3
6	100 “ “	5		9.8

It is evident that the border-line of ketonuria lies between the conditions in the two periods, the first showing small but distinctly abnormal amounts of acetone bodies, whereas with the larger amount of sugar, the acetone excretion is even smaller than observed by Lang when on full mixed diets. The ketogenic anti-ketogenic balance and the ratio point to the conclusion that the

<sup>1</sup>Lang (29), p. 463.

acetone bodies first appear when the molecular ratio, as here calculated, exceeds 1. In the period when ketonuria existed the ratio is 1.25, while it is 0.88 when ketonuria was absent.

The total energy exchange is estimated by Lang at 2,500 calories. On this assumption we may calculate the materials burned on the last day of each period as follows.

$$\begin{array}{rcl}
 \text{3rd day.} & 9.7 \text{ gm. of urine N} \times 26.5 & \overset{\text{calories}}{=} 257 \\
 & 60.0 \text{ gm. of sucrose} & = 258 \\
 & & \hline
 & & 515 \\
 2,500 - 515 & = 1,985 \text{ calories} & = 213 \text{ gm. of fat burned.} \\
 \text{6th day.} & 9.8 \text{ gm. of urine N} \times 26.5 & \overset{\text{calories}}{=} 260 \\
 & 100.0 \text{ gm. of sucrose} & = 380 \\
 & & \hline
 & & 640 \\
 2,500 - 640 & = 1,860 \text{ calories} & = 200 \text{ gm. of fat burned.}
 \end{array}$$

	Ketogenic.	Antiketo- genic.	Ratio.
	millimols	millimols	$\frac{K}{A}$
3rd day, 60 gm. of sugar.			
N, 9.7 gm.....	97	194	
Fat, 213 gm.....	734	121	
Sucrose, 60 gm. (63 gm. invert sugar).....		350	
Total.....	831	665	1.25
6th day, 100 gm. of sugar.			
N, 9.8 gm.....	98	196	
Fat, 200 gm.....	688	114	
Sucrose, 100 gm. (105 gm. invert sugar).....		584	
Total.....	786	894	0.88

*Data of Ascoli and Preti (30).*—A normal man, weighing at the start about 59 kilos and whose total energy requirement the authors estimated at 33 calories per kilo, was maintained on fat and protein for a period of several months. With changes in the amounts of food protein and carbohydrate the acetone body excretion varied from about 1 to 13 gm. expressed as hydroxybutyric acid.

Toward the end of the experiment increasing amounts of carbohydrate were added and with 45 gm. the excretion of acetone fell to 130 mg. and of hydroxybutyric acid to 100 mg. or less per day. Taking this period as representing the border-line of ketosis, I have calculated the ketogenic antiketogenic balance (day of February 9) as follows.

$$54 \text{ kilos} \times 33 \text{ calories} = 1,782 \text{ calories for total energy exchange}$$

$$\text{Urine nitrogen, 12.2 gm.} \times 26.5 \text{ calories} = \begin{array}{r} 323 \text{ calories from protein} \\ 1,459 \text{ calories from fat and CH} \end{array}$$

$$45 \text{ gm. of glucose} \times 3.76 \text{ calories} = \begin{array}{r} 169 \text{ calories} \\ 1,290 \text{ calories from fat} \end{array}$$

$$1,290 \div 9.4 = 139 \text{ gm. of fat burned.}$$

	Ketogenic.	Antiketo- genic.	Ratio.
	<i>millimols</i>	<i>millimols</i>	$\frac{K}{A}$
N, 12.2 gm.....	122	244	
Fat, 139 gm.....	477	79	
Glucose, 45 gm.....		250	
Total.....	599	573	1.04

For the preceding day, (February 8) with 30 gm. of glucose added to the diet, and when 220 mg. of acetone and about 200 mg. of hydroxybutyric acid were excreted, the balance may be calculated as follows.

$$\begin{array}{r} \text{Total} \quad \quad \quad \text{calories} \\ \quad \quad \quad 1,782 \\ 13 \text{ gm. of urine, N} \times 26.5 = \quad 345 \\ \hline \quad \quad \quad 1,437 \end{array}$$

$$30 \text{ gm. of glucose} \times 3.76 \quad 113$$

$$\text{From fat} \quad \quad \quad = 1,324 \div 9.4 = 141 \text{ gm. of fat.}$$

	Ketogenic.	Antiketo- genic.	Ratio.
	<i>millimols</i>	<i>millimols</i>	$\frac{K}{A}$
N, 13 gm.....	130	260	
Fat, 141 gm.....	483	80	
Glucose, 30 gm.....		167	
Total.....	613	507	1.21

It would appear from these data that the mixture being metabolized when the subject was on the border-line of ketonuria was such as to supply approximately equimolecular amounts of ketogenic and antiketogenic substances.

*Dietary of Eskimos.*—Reference is occasionally made to the Eskimo as illustrating an acquired ability to oxidize large amounts of fat with little or no carbohydrate. But when one calculates the ketogenic antiketogenic balance of their dietaries the explanation of their reputed tolerance for fat becomes evident. The calculation by Krogh (31) of the data collected by Rink may be cited as representing such dietaries, according to which the average daily diet of an adult Eskimo contains 282 gm. of protein, 135 gm. of fat, and 54 gm. of carbohydrate. The metabolism of such a mixture would not be expected to lead to acetone body production, the ratio being only 0.7. Even without all carbohydrate, the subject could probably remain practically free from acetonuria, the antiketogenic value of the protein at least equalling the ketogenic value of protein and fat, as shown below.

*Eskimo Dietary (Krogh).*

	Ketogenic.	Antiketogenic.	Ratio.
	<i>gm. molecule</i>	<i>gm. molecule</i>	$\frac{K}{A}$
Protein, 282 gm. = 45.3 gm. N.....	0.453	0.906	
Fat, 135 gm.....	0.452	0.077	
Carbohydrate, 54 gm.....		0.300	
Total.....	0.905	1.283	0.7

*Subject, H. S. K.*—We have carried out experiments similar to those cited from the literature, with in general the same results. Only one need be included in this presentation, and this will be stated briefly. The subject was a patient in the surgical service of the Barnes Hospital and was studied in the Metabolism Ward over a period while awaiting operation for a suspected brain tumor. His complaint was gradual loss of vision; no abnormality of metabolism was detected, and as far as the subject of this paper is concerned, the patient may be considered normal. Age 56, body weight 82 kilos. He remained in bed throughout

TABLE II.

*Subject H. S. K.*

Date.	Diet.			Urine N.	Metabolized.			Ketogenic.			Antiketogenic.			Ratio.	Total hydroxy- butyric acid excreted. gm.
	Pro- tein.	Fat.	C H		Pro- tein.	Fat.	C H	Pro- tein.	Fat.	Total.	Pro- tein.	Fat.	C H	Total.	
	gm.	gm.	gm.		gm.	gm.	gm.	milli- mols	milli- mols	milli- mols	milli- mols	milli- mols	milli- mols	milli- mols	
Dec. 14 (17.5 hrs.)	20	68	27	8	51	122	27	80	407	487	160	69	150	379	1.28
" 15	50	179	38	10.8	69	172	38	108	574	682	215	98	211	524	1.3
" 16	50	179	63	(10.0)	64	164	63	100	548	648	200	93	350	643	1.0
" 17	48	179	75	8.8	56	162	75	88	542	630	176	92	417	685	0.9
" 18	Hospital diet.														0.08

the experiment. The resting metabolism was determined daily with the Benedict spirometer unit apparatus (not the portable), by Dr. L. P. Gay, to whom I am indebted for the results.

After about 60 hours fasting, the patient was given for 2 days a diet representing about 10 per cent over his resting metabolism and containing about 10 per cent of the total calories in the form each of carbohydrate and protein, and 80 per cent as fat. It was expected that this diet would cause the continued excretion of small amounts of acetone bodies, which proved to be the case. The urines were collected in short periods and each analyzed for total nitrogen, acetone + acetoacetic acid, and hydroxybutyric acid.

On the 3rd day of the diet at 5.30 p.m., and on the 4th day additional starch was given and the total acetone body excretion dropped immediately from about 20 mg. of acetone per hour to about 4 mg. per hour.

The results are summarized in Table II. In making the calculations of the ketogenic balance the following assumptions are made: (1) That the total energy exchange for the day was 10 per cent higher than the average determined rate of the resting metabolism; (2) that stored glycogen was exhausted and not available; (3) that the carbohydrate fed was burned; and (4) that the protein metabolized is represented by the nitrogen excretion.

The results show that the ketogenic ratio at the time of small but definite acetone body excretion was with this case, like the preceding subjects, in the neighborhood of 1.

If one reviews the data calculated from the experiments of Zeller, Lang, Ascoli and Preti, the Eskimo dietary, and the experiment with subject H. S. K. it is found that metabolism of the mixtures of fat, carbohydrate, and protein in the proportions shown in Table III led to little or no acetone body excretion, and therefore, are examples of metabolic mixtures at the border-line of ketosis and ketonuria.

From these data one is led to draw the conclusions: (1) that the production and excretion of acetone bodies are dependent upon the *relative* amounts of protein, fat, and carbohydrate in the mixture being metabolized at the time; (2) that, as here calculated, the border-line molecular ratio between ketogenic and

antiketogenic factors is 1; and (3) that such diets as supply from carbohydrate at least 10 per cent, from protein about 10 per cent, and from fat not more than 80 per cent of the energy required by the subject, will produce little or no ketonuria.

If the argument being developed in this paper is sound, it should be possible to apply the method of calculation not merely to the border-line of ketosis but to results from cases of extreme acidosis and ketonuria as well. I have made a number of such calcula-

TABLE III.  
*Calculated Metabolic Mixture near Threshold of Ketonuria.*

Author or subject.	Per cent of total calories from:			Ratio.	Total hydroxy-butyric acid excreted.
	Fat.	Protein.	Carbohydrate.		
				$\frac{K}{A}$	gm.
Zeller.....	82	4	14	1.23	+
Lang.....	80	10	10	1.25	0.36
	75	10	15	0.88	0
Ascoli and Preti.....	72	18	10	1.0	$\pm 0.3$
	74	19	6	1.2	$\pm 0.6$
Eskimo dietary.....	48	44	8	0.7	0(?)
H. S. K.....	78	14	8	1.28	0.74
	78	14	8	1.3	0.77
	75	13	13	1.0	0.36
	74	11	15	0.9	0.18

tions. In most instances they show what is already indicated by a comparison of the  $\frac{K}{A}$  ratios and the amounts of total acetone bodies excreted, as given above in Table III; namely, that although the border-line of ketosis falls approximately at a ratio of 1, when the ratio is greater than 1 the excretion expected from the computation often greatly exceeds the amounts actually found. For example, when subject H. S. K. excreted about 0.7 gm. of total hydroxybutyric acid he had a calculated ratio of 1.30, and if the calculations and underlying assumptions were altogether



correct he should have excreted an amount of hydroxybutyric acid corresponding to the excess of ketogenic over antiketogenic molecules, which on this day amounted to 158 millimols, or  $0.158 \times 104 = 16.4$  gm. total hydroxybutyric acid. The realization thus falls short of the expectation and indicates that some of the assumptions are wrong. The explanation may, of course, be that some ketogenic substance may be oxidized without the intervention of ketolytic substance, but such a conclusion should be accepted only as a last resort, and if it is not possible to interpret the results without such additional assumption.

The necessary corrections and modifications will doubtless require extended investigation and it is not proposed to make such an attempt here; but it may be stated that the corrections required appear to be necessary rather for the relative values assigned to the antiketogenic factors and not for the ketogenic, which latter there is reason to believe are approximately correct.

As evidence for the last statement and in general confirmation of our hypothesis a recalculation is presented of the very interesting case of severest diabetes, "Cyril K," studied and reported in admirable detail by Gephart, Aub, Du Bois, and Lusk (32).

*Severest Diabetes. "Cyril K" of Gephart, Aub, Du Bois, and Lusk.*

During a few days this subject was a total diabetic and the authors state that "during this period . . . all the glucose derivable from protein and all the beta-oxybutyric acid formed as an end-product of the oxidation of fat is completely eliminated in the urine." They further state that "a considerable origin of beta-oxybutyric acid from (amino acids of protein) is not indicated." But according to my calculations of their data, the amounts of total hydroxybutyric acid excreted correspond fairly well with the expectations based on the assumptions, stated earlier in this paper, as to the ketogenic value of protein as well as fat.

Due to the fact that the subject during several days metabolized very little of the sugar-forming material from glycerol as well as from protein, he was thus almost wholly deprived of antiketogenic effect, and in consequence virtually all of the ketogenic mole-

cules catabolized appeared as acetone and acetoacetic and hydroxybutyric acids. And since antiketogenic effect is almost absent, errors in its calculation are also nearly excluded and we are enabled to test the accuracy of the computation of ketogenic factors alone. The results are on the whole quite in harmony with our estimates of the relative ketogenic values of fat and protein.

The statement above quoted from Lusk and his coworkers, as to the origin of hydroxybutyric acid from protein, is based upon calculations from the respiratory quotients, which may be not wholly reliable in severe acidosis. For this reason it has seemed preferable in my computations of these data not to depend upon the respiratory quotients. The method of making the calculations may be illustrated by the following example representing the first day (December 15) on which the subject was studied in the calorimeter.

The hourly metabolism is given as 81.9 calories which for 24 hours would be 1,966 calories. The total acetone body excretion was about 80 gm. of hydroxybutyric acid, (70.9 gm. of hydroxybutyric + 5.61 gm. of acetone and diacetic acid) allowing for the excretion of a few gm. of acetone in the breath. The caloric value of this amount,  $80 \times 4.69 = 375$  calories, is added to the heat produced, giving 2,341 calories. The heat produced from the non-carbohydrate quota of protein is, 36.4 gm. of urine nitrogen  $\times 12.97$  calories = 472 calories leaving 1,869 calories derived from the combustion of fat and carbohydrate, including glucose from protein. The amount of sugar burned is estimated as follows:

Urine glucose — food CH = glucose excreted from protein and glycerol

$$167.9 \text{ gm.} - 24 \text{ gm.} = 143.9 \text{ gm.}$$

Glucose possible from protein =  $36.4 \times 3.6 = 131$  gm.

“Extra glucose,” from glycerol = 12.9 gm.

According to this calculation the subject failed to use, and excreted, not only all of the glucose from protein but 12.9 gm. from glycerol. The caloric equivalent of the latter must be added to 1,869 giving 1,917 calories, equivalent to 203 gm. of fat which was metabolized (incompletely). From these data the ketogenic balance is tabulated.

	Ketogenic.	Antiketogenic.	Remarks.
	millimols	millimols	
N, 36.4 gm. . .	364	0	All glucose excreted.
C H. . . . .	0	0	No food C H burned.
Fat, 203 gm. .	696	45	116—(12.9 × 5.56) for glucose excreted from glycerol.
Total. . . . .	1,060 — 45 = 1,015 excess ketogenic.		

$1.015 \times 104 = 105$  gm. of total hydroxybutyric acid expected.

About 80 gm. were actually excreted. It will be noted that glucose corresponding to all of the fat catabolized is 21 gm. of which 12.9 gm. or 61 per cent were excreted. This condition continued during the next 2 days after which, with the onset of fasting, there was a sudden, though small, increase in the sugar-burning power which, coinciding with the fall in total metabolism and the amount of fat burned, resulted in the gradual approach toward ketogenic balance, until on the last day only 11 gm. of hydroxybutyric acid were excreted.

The details of the calculations for other days are given in Table IV and from this the figures representing the ketogenic balance are brought together in Table V. From these data it will be seen that the basis used for the calculation of the total ketogenic and antiketogenic factors accounts approximately for the total hy-

TABLE IV.  
*Results from Severe Diabetic, "Cyril K."*

Date.	Food C H.	Urine N.	Urine glucose.	Possible glu- cose from protein (N×3.6).	Urine glucose — food C H.	Glucose from protein burned.	"Extra" glu- cose excre- ted (from glycerol).	Total hydroxy- butyric acid excreted.	
	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	calorie value
Dec. 15 . . . . .	24	36.4	167.9	131	143.9	0	12.9	80±	375
" 16 . . . . .	0.4	38.3	153.4	138	153	0	15.0	80±	375
" 17 . . . . .	0.4	36.3	140.3	130.7	140	0	9.3	87+	408
" 18 . . . . .	Fast.	20	55.1	72	55	17	0	58.5+	275
" 19 . . . . .	"	16.7	44.3	60	44.3	16	0	56.8+	267
" 20 . . . . .	6	14.1	35.3	50.8	29.3	21.5	0	41.2+	193
" 21 . . . . .	6.6	14.4	39.7	51.8	33.1	18.7	0	26.2+	122
" 22 . . . . .	11.4	18.3	26.0	65.9	14.6	51.3	0	11.0+	52

TABLE IV—*Concluded.*  
Ketogenic Balance of "Cyril K."

Date.	Total calories liberated.		Calories from			Fat catabolized	Ketogenic millimols.			Antiketogenic millimols.		
			Non C H quanta of protein.	Protein glucose burned.	Fat burned		Protein.	Fat.	Total.	Protein.	Fat.	Total.
	per hr.	per 24 hrs.		gm.								
Dec. 15.....	81.9	1,966	472	0	1,917	203	364	696	1,060	0	116-72=44	44
“ 16.....	76.4	1,833	497	0	1,767	187	383	642	1,025	0	107-83=24	24
“ 17.....		(1,800)	471	0	1,772	187	363	642	1,005	0	107-52=55	55
“ 18.....	73.2	1,757	260	64	1,708	181	200	620	820	94	103	197
“ 19.....		(1,670)	216	60	1,661	175	167	600	767	89	89	178
“ 20.....	66.3	1,590	183	81	1,519	160	141	549	690	120	120	240
“ 21.....		(1,550)	187	70	1,415	150	144	515	659	104	104	208
“ 22.....	62.8	1,507	237	193	1,129	119	183	408	591	285	68	353

TABLE V.

## Summary of Ketogenic Balance of "Cyril K."

Comparison of hydroxybutyric acid excretion with calculated expectation.

Date.	Excess ketogenic.	Expected hydroxybu- tyric acid.	If protein and glycerol act as triose.		Hydroxybu- tyric acid actually excreted.
			Excess ketogenic.	Expected hydroxybu- tyric acid.	
	<i>millimols</i>	<i>gm.</i>	<i>millimols</i>	<i>gm.</i>	<i>gm.</i>
(1)	(2)	(3)	(4)	(5)	(6)
Dec. 15.....	1,016	105	972	101	80±
" 16.....	1,001	104	977	102	80±
" 17.....	950	99	895	93	87+
" 18.....	623	65	426	44	58+
" 19.....	589	61	411	43	57+
" 20.....	450	47	210	22	41+
" 21.....	451	47	243	25	26+
" 22.....	238	25	0	0	11+
Totals.....		559		436	440+

droxybutyric acid formed and excreted. The greatest discrepancy is about 25 gm. and this would become less if the amount of acetone exhaled were included. The figures indicate, in the writer's opinion the following points: (1) that each molecule of fatty acid gives rise to one molecule of acetoacetic acid. If more than one molecule were formed the actual excretion would have greatly exceeded the expectation. (2) That protein is markedly ketogenic, the production from fat (fatty acid) alone being quite inadequate to account for the amounts of acetone bodies formed. (3) No other source of acetone bodies is indicated, and (4) consequently, the method of calculating the ketogenic factors is approximately correct.

But as already suggested, the method of calculating the antiketogenic influences is less satisfactory. In Table V are given the expected excretion (formation) of total acetone bodies, as gm. of hydroxybutyric acid, calculated (in Column 3) on the assumption used in the preceding calculations, that the active antiketogenic (ketolytic) substance from protein and glycerol as well as from carbohydrate is a six carbon derivative of glucose; and in Column 5 the expected excretion is calculated on the assumption that glycerol and the antiketogenic amino-acids are active in the form of three carbon derivatives. In the latter case the antiketogenic value of protein and of fat would be twice the values given on page 457. While the writer prefers to reserve an opinion on this point, it appears that the latter assumption more nearly coincides with the facts. The total amount excreted during the period (exclusive of acetone in breath, and of acetone and acetoacetic acid on 6 of the 8 days) was 440 gm. The total expected on the first assumption is 559 gm. and on the second assumption 436 gm. In view of the fact that the total metabolism and the amount of fat actually catabolized probably were in excess of the resting periods in calorimeter, which were used as the basis of calculation, one would expect the amounts of acetone bodies excreted to be greater and not less than the calculated amounts. From this point of view the second assumption is the more likely. Also it may be recalled that the *in vitro* ketolytic effect of glycerol is probably the same as an equimolecular amount of glucose. The question is being further investigated.

In view of the many opportunities for error, the moderately close agreement by either method is considered rather remarkable, and is at least strong evidence in support of the underlying conception as to the nature of antiketogenesis.

#### SUMMARY AND CONCLUSIONS.

Starting with the hypothesis that the property possessed by carbohydrate and other substances of preventing the appearance of acetone bodies (the phenomenon of "antiketogenesis"), is due to a chemical reaction in the body between definite and constant amounts of "ketogenic" and "antiketogenic" compounds, analogous to the "ketolytic" reaction between acetoacetic acid and glucose described in the preceding paper, a trial method has been developed for the calculation of the molecular amounts of ketogenic and of antiketogenic substances derivable from protein, fat, and carbohydrate.

The application of this calculation to various subjects who were excreting small amounts of acetone bodies, indicates that the general hypothesis is correct and that the minimum molecular ratio of ketogenic to antiketogenic substances for the avoidance of ketonuria in different human subjects is 1.

This conclusion is confirmed by the calculation of data from a case of "total" diabetes with extreme acidosis, the total hydroxybutyric acid excretion being approximately accounted for and in fair agreement with the calculated expectation.

There appears to be no reason for believing that any factors other than those concerned with this ratio influence acetone body formation. No difference is to be expected in the behavior of different human subjects, whether normal or diabetic, as regards acetone body formation, except as accounted for by the excess of ketogenic over antiketogenic molecules in the mixture being catabolized.

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## A SIMPLE TECHNIQUE FOR THE DETERMINATION OF CALCIUM AND MAGNESIUM IN SMALL AMOUNTS OF SERUM.

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(Received for publication, June 1, 1921.)

### *Method.*

1 or 2 cc. of serum are measured into an ordinary 15 cc. graduated centrifuge tube containing 2 cc. of water. 1 cc. of a saturated solution of ammonium oxalate is then added and the sample mixed. The mixture is allowed to stand for half an hour. The volume is then made up to 6 cc. with distilled water and the sample again mixed. The tube is centrifuged at 1,300 revolutions per minute for 10 minutes. All but 0.3 cc. of the supernatant fluid is syphoned off. The precipitate is mixed with the remaining fluid and 2 per cent ammonia (2 cc. of concentrated ammonia to 98 cc. of water) is added up to 4 cc. and mixed. The tube is then centrifuged for 5 minutes. All but 0.3 cc. of the supernatant fluid is again syphoned off. The last step is repeated twice, making three washings in all. The tube is centrifuged for 5 minutes after each washing. After the last washing the supernatant fluid is syphoned off. The crystals are suspended in the residual liquid and dissolved in 2 cc. of approximately  $\times$  sulfuric acid, heated in the water bath for a few minutes, and then titrated to a definite pink color, which persists for at least 1 minute, with 0.01  $\times$  potassium permanganate. This is delivered from a micro-burette graduated in 0.02 cc.

### *Calculations.*

The number of cc. of 0.01  $\times$  potassium permanganate used — the blank (volume of 0.01  $\times$  potassium permanganate required to produce the same intensity of color in an equal volume of water)  $\times$  0.2 = the number of mg. of calcium in the sample.

*Reagents.*

The only reagent that must be quantitatively accurate is the 0.01 N sodium oxalate (Sørensen). This is used to standardize the permanganate. An 0.1 N sodium oxalate is prepared in the usual way. Solution of the oxalate is facilitated by the addition of 5 cc. of concentrated sulfuric acid. The solution is then diluted 10 times to make an 0.01 N sodium oxalate. The latter solution will remain unchanged for several months; the former indefinitely. The other reagents are prepared in the usual way.

*The Magnesium Determination.*

5 cc. of the supernatant fluid from the calcium determination corresponding to 1.66 cc. of serum are measured into a 30 cc. beaker, 1 cc. of  $(\text{NH}_4)_2\text{HPO}_4$  solution is added and then 2 cc. of concentrated ammonia. The next day the sample is filtered through a well packed Gooch crucible, washed ten times with 5 cc. of 10 parts of concentrated ammonia to 90 parts of water, then twice with 95 per cent alcohol made alkaline with ammonia. The crucible is returned to the beaker and dried for a few minutes at 80° C. in the oven.

10 cc. of 0.01 N HCl are added to the crucible and after a few hours the entire material is transferred to a test-tube, centrifuged, and 5 cc. of the supernatant fluid are measured into a flat bottomed colorimeter tube graduated for 10 cc., which contains 2 cc. of the iron thiocyanate solution. The volume is then made up to 10 cc. with 0.01 N HCl, a rubber stopper inserted, and the fluid mixed. A series of standards is prepared by adding varying amounts of a known  $\text{NH}_4\text{MgPO}_4$  solution to the thiocyanate solution and bringing the volume up to 10 cc. as in the unknown samples. The color is compared by looking through the entire length of the liquid column against a white background.

*Calculation.*—The calculation is the same as in the original method:  $\text{Reading (cc. of standard solution)} \times 0.01 \times 2 \times \frac{6}{5} \times 50 = \text{mg. of magnesium in 100 cc. of serum when 2 cc. of serum are used.}$

*Preparation of Reagents.*

*Ammonium Magnesium Phosphate Standard.*—This solution is made by dissolving 0.102 gm. of air-dried magnesium ammonium

phosphate ( $\text{MgNH}_4\text{PO}_4 \cdot 6\text{H}_2\text{O}$ ) in 100 cc. of 0.1 N hydrochloric acid and diluting to 1 liter with water. Of this solution 1 cc. is equivalent to 0.01 mg. of magnesium. Magnesium ammonium phosphate loses water of crystallization when heated and must therefore be dried at room temperature. Commercial preparations of the salt are generally unreliable; it should be prepared by precipitation of pure solutions.<sup>1</sup>

*Ammonium Phosphate Solution.*—This solution is made as follows: 25 gm. of  $(\text{NH}_4)_2\text{HPO}_4$  are dissolved in 250 cc. of  $\text{H}_2\text{O}$ . 25 cc. of concentrated ammonia are added and the mixture is allowed to stand over night. The following day it is filtered, the filtrate is boiled to remove the excess of ammonia, cooled, and made up to 250 cc. This solution is diluted 5 times with water.

*The Ferric Thiocyanate Solution.*—The ferric thiocyanate solution is made from two solutions which are mixed an hour before use. Solution A is 0.3 per cent ammonium thiocyanate. Solution B is 0.3 per cent ferric chloride, made up from the salt with its contained water of crystallization, adding a few drops of acid, if necessary, to clear the solution. 5 cc. portions of Solutions A and B are mixed and the whole is diluted to 40 cc. with water.

*10 Per Cent Ammonia.*—100 cc. of concentrated ammonia are diluted to 1 liter.

### *Protocols.*

Solutions of blood salts containing calcium and magnesium in concentrations comparable to those found in serum and as high as 30 mg. of phosphorus per 100 cc. of solution in the form of *o*-phosphoric acid have been analyzed by this method and the calcium recovered within 5 per cent of the amount actually present (Table I).

Table II gives the results of a series of comparative determinations on normal and pathological sera performed by this method and by another method previously described by us, the accuracy of which has been established (1).

Table III gives the results of the estimation of magnesium in the supernatant fluid derived from the determination by either calcium method. The figures obtained show excellent agreement.

<sup>1</sup> Jones, W., *J. Biol. Chem.*, 1916, xxv, 87.

## DISCUSSION.

The use of ammonium oxalate for the precipitation of calcium is a well known procedure. Its application for the direct determination of calcium in unashed serum was used by Pribram (2) as early as 1871 and more recently by both de Waard (3) and Clark

TABLE I.  
*Analyses of Samples of Solution B.*

Specimen No.	Calcium.	
	Found.	Present.
	mg.	mg.
1	0.102	
2	0.098	
3	0.096	
Average.....	0.098	0.100
4	0.198	
5	0.206	
6	0.200	
Average.....	0.202	0.200
7	0.294	
8	0.290	
9	0.302	
Average.....	0.296	0.300

## Composition of Solution B.

NaCl.....	7.739 gm.
Na <sub>2</sub> HPO <sub>4</sub> + 2 H <sub>2</sub> O.....	2.005 "
KCl.....	0.453 "
CaCO <sub>3</sub> .....	0.250 "
MgSO <sub>4</sub> + 7 H <sub>2</sub> O.....	0.189 "
Concentrated HCl.....	10.0 cc.
Water to.....	1,000.0 "

(4). The former has reported only a few determinations on cow's serum. These are 10 to 15 per cent lower than the results obtained by Meigs, Blatherwick, and Cary (5), Halverson and Bergeim (6), and Marriott and Howland (7) on material derived from similar sources. Thus de Waard reports the finding of

8½ mg. of calcium per 100 cc. of cow's serum whereas all the investigators above mentioned have found approximately 10 mg. in the same volume of serum.

Clark (4) has briefly described methods for the direct determination of calcium in plasma and whole blood, using ammonium

TABLE II.

*Comparative Calcium Determinations on Serum by the Simplified Technique and the Authors' Original Method.*

Specimen No.	Diagnosis.	Ca per 100 cc. of serum.	
		Simplified method.	Authors' original method.
		mg.	mg.
1	Normal adult.	10.0	9.6
2	" "	10.5	10.6
3	" "	9.6	9.2
4	" "	9.6	9.5
5	" "	9.9	9.9
6	" "	9.5	9.7
7	" "	10.3	10.1
8	" "	10.3	10.3
9	" "	9.8	10.1
10	" "	10.0	10.0
11	" 11 years old.	9.7	9.7
12	" 9 " "	10.5	11.1
13	Scurvy.	10.2	9.7
14	Diabetes mellitus.	10.7	10.5
15	Pyelonephritis.	10.9	10.3
16	Rickets.	9.8	9.5
17	"	8.0	7.7
18	"	7.8	7.4
19	Tetany.	7.5	7.5
20	"	7.3	7.7
21	"	6.2	6.3
22	"	5.7	5.5
23	Chronic nephritis.	7.3	7.8

oxalate which has been titrated to a pH of 7.4 as the precipitating reagent. No figures are published to support the claims made for these methods. In a subsequent communication dealing with the effect of the administration of calcium salts to rabbits, results are reported for the calcium content of plasma and whole blood but the methods used are not described.

In a subsequent paper we shall discuss in detail the sources of error in the different methods that have been described for the determination of calcium in serum, plasma, and blood. In the present communication we merely desire to place on record a simplified technique for the quantitative determination of this element in small amounts of serum which has been in use in this laboratory for almost a year and has given satisfactory results when tested on a large series of both normal and pathological sera. Some of these results are published in Table II.

TABLE III.  
*Determination of Magnesium in Serum.*

Name.	Age.	Diagnosis.	Mg per 100 cc. of serum.	
			Simplified method.	Authors' original method.
			<i>mg.</i>	<i>mg.</i>
E. B.	1 yr.	Normal.	1.8	1.8
L. B.	1 "	Tetany(treated).	1.8	1.8
L. B.	1 "	" "	2.4	2.1
B. K.	Adult.	Normal.	2.1	2.2
S. G. R.	"	"	2.2	2.1
F. F. T.	"	"	2.1	2.5
B. K.	"	"	2.1	1.9
F. F. T.	"	"	2.1	2.2
B. B.	"	"	2.3	2.3
G. M.	2 yrs.	Rickets.	2.2	2.2
R. G.	18 mos.	Epilepsy.	1.8	1.8
C. L. D.	14 "	Scurvy.	3.0	2.8

#### CONCLUSIONS.

A simple and rapid technique for the quantitative determination of calcium in 1 or 2 cc. of serum has been described. The supernatant fluid from this determination may be used for the quantitative determination of magnesium. These methods are accurate to within  $\pm 5$  per cent of the amount of calcium and magnesium present.<sup>2</sup>

<sup>2</sup> Dr. von Meysenbug (personal communication) has compared this calcium method with that of Lyman and obtained excellent agreement.

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## VITAMINE STUDIES.

### VIII. THE EFFECT OF HEAT AND OXIDATION UPON THE ANTISCORBUTIC VITAMINE.\*

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(Received for publication, June 7, 1921.)

During the summer of 1918 experiments were conducted in this laboratory which indicated that rhubarb juice and orange juice could be boiled for 15 minutes without appearing to lose their antiscorbutic potency. At the time, we were inclined to the belief that the natural acidity of these juices acted in a protective manner. Delf,<sup>1</sup> working with juices extracted from fresh cabbages, Swedish turnips, and oranges, made similar observations. She suggested that the stability of the antiscorbutic vitamine, to temperatures above 100°C., might be due to the absence of air.

Other workers<sup>2, 3, 4, 5,</sup> have studied the influence of acidity and alkalinity upon the stability of the antiscorbutic vitamine and are in general agreement that the vitamine is stable in acid solution, while alkalinity tends to destroy it.

Rossi<sup>6</sup> has shown that guinea pigs develop scurvy and die in about 20 days when subsisting upon a diet of hay and oats which

\* Published with the approval of the Director as Paper No. 262, Journal Series, Minnesota Agricultural Experiment Station. A portion of the expense of this investigation was defrayed by a grant from the Graduate School, University of Minnesota.

<sup>1</sup> Delf, E. M., *Biochem. J.*, 1920, xiv, 211.

<sup>2</sup> Weill, E., and Mouriquand, G., *J. physiol. et path. gén.*, 1918, xvii, 849.

<sup>3</sup> Hess, A. F., and Unger, L. J., *J. Biol. Chem.*, 1919, xxxviii, 293.

<sup>4</sup> Harden, A., and Zilva, S. S., *Lancet*, 1918, ii, 320.

<sup>5</sup> Lamer, V. K., Campbell, H. L., and Sherman, H. C., *Proc. Soc. Exp. Biol. and Med.*, 1920-21, xviii, 122.

<sup>6</sup> Rossi, G., *Arch. fisiol.*, 1918, xvi, 125, cited in *Chem. Abstr.*, 1920, xiv, 1844.

has been subjected to open sterilization. When the same materials are sterilized in hermetically sealed jars guinea pigs grow well and remain healthy for 60 days or more.

Recently, Hess and Unger<sup>7</sup> and Zilva<sup>8</sup> have shown in a more conclusive manner that oxidation has a decidedly destructive action upon solutions containing the antiscorbutic vitamine. The last mentioned writer also observed that carbon dioxide did not inactivate lemon juice, while air possessed a very destructive action. This agrees with our findings with regard to the influence of carbon dioxide and air upon the antiscorbutic potency of heated milks.<sup>9</sup>

Ellis, Steenbock, and Hart<sup>10</sup> have shown that the antiscorbutic vitamine is susceptible to oxidizing agents although they were unable to prevent its destruction in cabbage when this foodstuff was desiccated for 35 hours at 65°C. in an atmosphere of carbon dioxide. The last mentioned observation is difficult to explain unless the destructive action was brought about by intracellular oxidation during the drying process.

#### EXPERIMENTAL.

The experimental technique in this experiment was similar to that described in previous papers published from this laboratory, with the exception that the scorbutic diet consisted of unhulled oats (60 per cent) and chopped alfalfa hay (40 per cent). The alfalfa hay was autoclaved for 30 minutes at 15 pounds pressure and dried before mixing with the oats. 3 cc. of orange juice, treated in various ways, as indicated in Table I, were fed (daily) to each animal. Hydrogen peroxide was used as the oxidizing agent.

In all cases, where hydrogen peroxide was used, 10 cc. of hydrogen peroxide (3½ per cent) were added for each 100 cc. of fresh orange juice. Those samples of orange juice which did not receive

<sup>7</sup> Hess, A. F., and Unger, L. J., *Proc. Soc. Exp. Biol. and Med.*, 1920-21, xviii, 143.

<sup>8</sup> Zilva, S. S., *Lancet*, 1921, i, 478.

<sup>9</sup> Anderson, E. V., Dutcher, R. A., Eckles, C. H., and Wilbur, J. W., *Science*, 1920-21, liii, 446.

<sup>10</sup> Ellis, N. R., Steenbock, H., and Hart, E. B., *J. Biol. Chem.*, 1921, xlv, 367.

treatment with hydrogen peroxide received 10 cc. of distilled water in order to make all samples comparable as to volume.

The pasteurized samples (63°C.) were heated in closed flasks in a water bath, while the boiled samples were boiled under reflux condensers to prevent loss by evaporation. All samples were prepared at the same time and under the same conditions. Sufficient quantities were prepared at one time to last for 1 week. After treatment all samples were cooled in running water and placed in a refrigerator. The containers were made of Pyrex glass, tightly stoppered to exclude the air.

TABLE I.  
*Experimental Diets.*

Experi- mental group.	Diet.
I	Basal diet + 3 cc. of raw orange juice.
II	" " + 3 " " orange juice + H <sub>2</sub> O <sub>2</sub> (room temperature).
III	" " + 3 " " " " heated for 30 minutes (63°C.).
IV	" " + 3 " " " " + H <sub>2</sub> O <sub>2</sub> heated for 30 minutes (63°C.).
V	Basal diet + 3 cc. of orange juice boiled for 30 minutes.
VI	" " + 3 " " " " + H <sub>2</sub> O <sub>2</sub> boiled for 30 minutes.

#### DISCUSSION.

The results of the experiments are indicated in Charts I, II, and III. All the animals receiving 3 cc. of raw orange juice (Group I, Chart I) grew well and No. 357 gave birth to healthy twins. Group II (Chart I), which received orange juice treated with hydrogen peroxide at room temperature, did not grow quite as well as those in Group I and they were in much poorer physical condition, tending to softness or flabbiness. Animals 366 and 367 also gave birth to twins but the young were born dead in both instances. Comparison of the animals in Groups III and IV (Chart II) shows that pasteurization of the orange juice (Group III) had no detrimental effect, for the growth curves are as good as those which received the unheated raw orange juice (Group I).

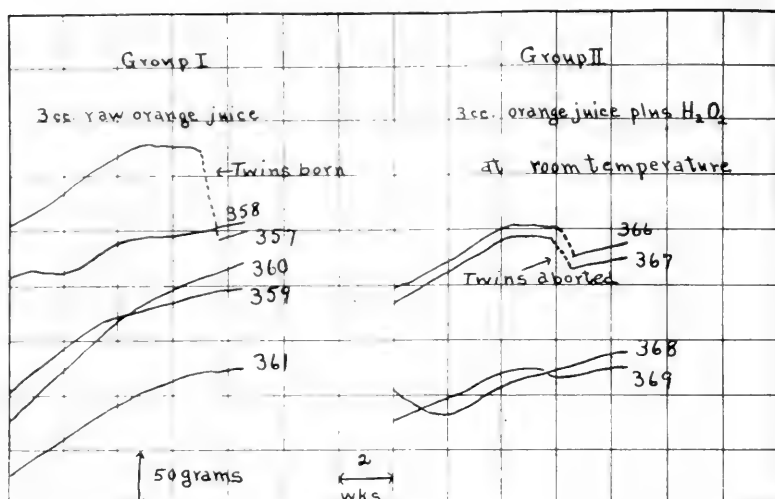


CHART I.

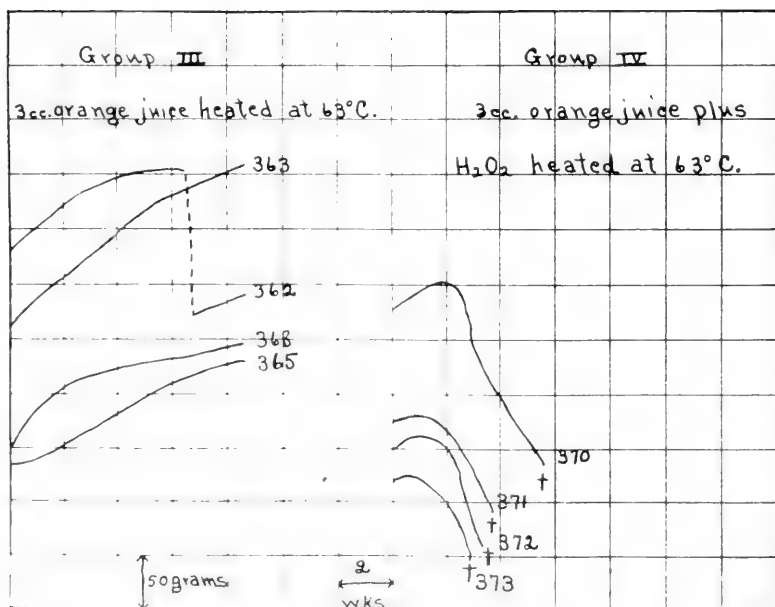


CHART II.

It would appear, therefore, that heat alone at 63°C. does not destroy the antiscorbutic vitamin. All the animals in Group IV, which had received orange juice that had been treated with hydrogen peroxide at 63°C., died with pronounced scurvy symptoms and lesions. These animals died within 3 to 6 weeks (average 4 weeks), while those in Group III were in excellent condition at the end of 2 months. Animal 362 (Group III) also gave birth to healthy twins, indicating that pasteurization had no harmful effect.

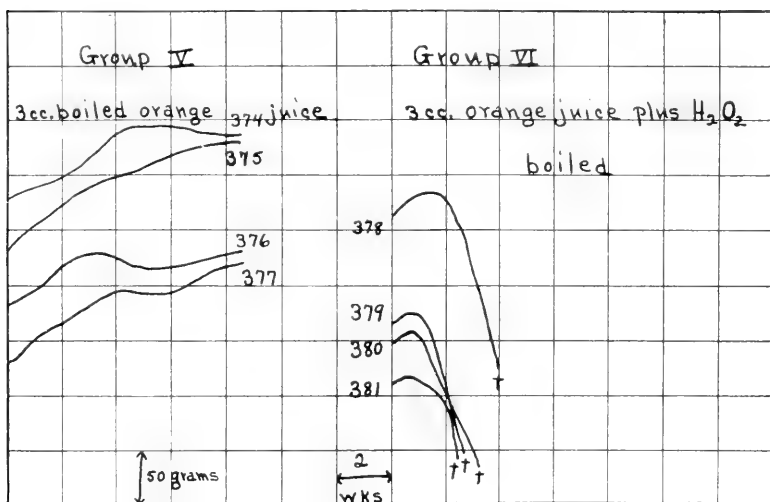


CHART III.

The growth curves of the animals in Group V (Chart III) indicate that boiling for 30 minutes has no destructive action upon the antiscorbutic vitamin, for all of the animals were in excellent physical condition at the end of the experimental period. The influence of hydrogen peroxide during the boiling process, however, is very marked. All the animals of Group VI (Chart III) died with scurvy in less than 30 days. This would indicate that the speed of the oxidative destruction of the vitamin is hastened as the temperature increases, appearing to conform to the recognized chemical laws regarding the influence of heat on chemical reactions.

## SUMMARY AND CONCLUSIONS.

1. The antiscorbutic vitamine is not destroyed by heating at pasteurization temperature ( $63^{\circ}\text{C}.$ ) for 30 minutes in closed vessels or by boiling ( $100^{\circ}\text{C}.$ ) for 30 minutes under reflux condensers.

2. Hydrogen peroxide possesses some destructive action when added to orange juice at room temperature and the destructive action is increased when the orange juice-hydrogen peroxide mixture is heated at  $63$  and  $100^{\circ}\text{C}.$

3. The antiscorbutic properties of orange juice are susceptible to oxidation but, in the absence of oxidizing agents, are stable to heat up to the boiling temperature of orange juice.

## A SIMPLE LABORATORY GAS METER AND AN IMPROVED HALDANE GAS ANALYSIS APPARATUS.

BY H. S. NEWCOMER.

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(Received for publication, May 23, 1921.)

Carpenter (1) has described in detail the methods which are available for the study of respiratory exchange in man. Of these the simplest is that of Douglas which depends upon the collection of a specimen of expired air in a rubber bag and the determination of its  $\text{CO}_2$  and  $\text{O}_2$  percentages in a Haldane gas analysis apparatus (2). Since the publication of Carpenter's work, several types of apparatus have appeared. They all determine the calorie production by methods of measuring the reduction in the oxygen volume in a closed system which includes the patient's respiratory tract. The methods, while simple, are not always satisfactory. In so far as they are limited to the determination of oxygen consumption, they fail to give as much information as is frequently desired concerning the gaseous metabolism. This paper describes some improvements in apparatus to be used in connection with methods similar to those of Douglas.

For the collection of expired air, one may use in addition to a suitable face mask and valves, either a calibrated spirometer (3) or a properly constructed rubber bag. A spirometer is less portable and more expensive. The rubber bag designed by Douglas furnishes an easy and portable means of collecting the expired air. For the measurement of the volume of its contents, it is customary to use a wet meter of the Bohr type. The Danish meters are very expensive, but those made in this country, while less elegant, are very reliable. They maintain vapor saturation of the expired air but there is no data available as to the error which they introduce by the absorption of the  $\text{CO}_2$  from the air

passed. They are not very portable and to read correctly must have the water level closely adjusted. In the case of the  $\frac{1}{10}$  cubic foot size, the volume read is 1 per cent low for each  $\frac{1}{16}$  inch rise of the water level above the mark set. The meters have a slow rate of flow and if the rate be more than about  $\frac{1}{3}$  cubic foot per minute there results a rise in the water level producing a contrac-

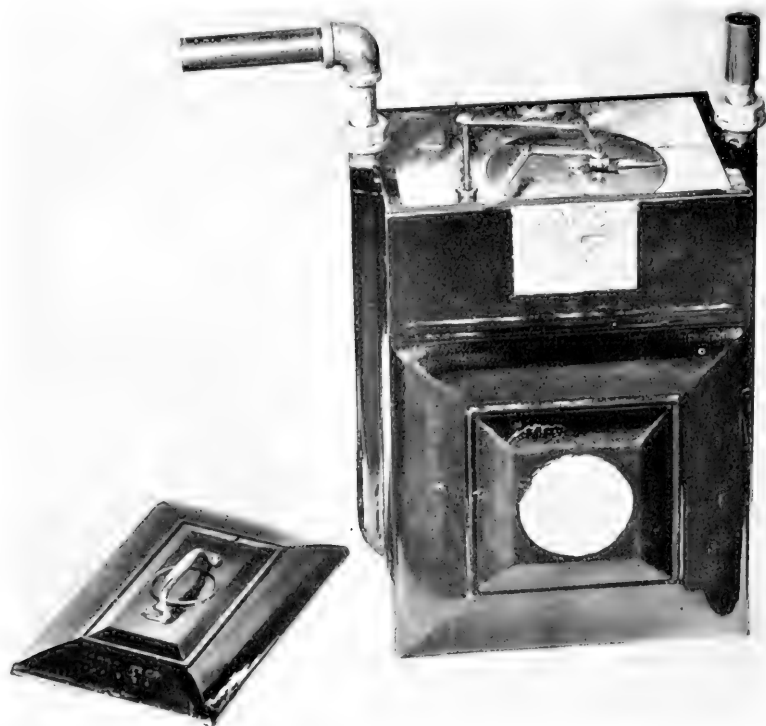


FIG. 1.

tion in the volume reading which may readily amount to 4 per cent. It is easy by increasing the rate, to cause the air to bubble through the meter and increase this error to 20 per cent or more.

An inexpensive and portable meter may be adapted from the ordinary five light gas service meter (Fig. 1). This meter has two soft oiled leather bellows in closed compartments, and works as a tandem reciprocating engine to rotate a vertical shaft at the



rate of one revolution per  $\frac{1}{8}$  cubic foot of gas passed. A worm drive from this operates the dial. The top of the meter may be removed, or the meter purchased without a top, and a graduated circle and hand placed on the vertical shaft. It then reads about 3,540 cc. per revolution. The factory adjustment of the meter is made to within about 1.0 per cent of this figure. The meter may be calibrated by displacing air from a large bottle with tap water.

The calibration of two such meters was done by delivering air from a 10 cubic foot standard meter prover of the spirometer type in one of the laboratories of the United Gas Improvement Company. The air was delivered under pressures varying from 0.5 to 2.5 mm. of Hg. The speed varied from 2 to 4 cubic feet a minute. A test performed with a standard cubic foot bottle, correct to one part in ten thousand and delivering air much slower, gave the same readings as the slowest of the above speeds. During numerous consecutive runs the variation in the meter reading was at any one pressure never more than two parts in one thousand. There was a variation due to pressure changes. Up to a speed of 2 cubic feet per minute the readings were constant. At 3 cubic feet per minute (1.6 mm. of Hg) the reading for 10 cubic feet (corrected) was 0.6 per cent low and at 4 cubic feet per minute (2.5 mm. of Hg) the reading was 1.0 per cent low. When calibrated at low speeds and used at higher speeds the meter reading can therefore be increased in these proportions. The decreased readings at higher pressures do not seem to be due to leakage through the reciprocating valves of the meter.

If the zero of the dial on the main shaft be taken as the top with the meter facing the operator, then Fig. 2 represents the calibration curves of two of these meters when run in series with a Bohr meter. The hand does not run evenly around the circle but varies by as much as 180 cc. from the expected value. The correction curves for the two instruments are about alike and the unevenness is a part of the design of the apparatus. A calibration curve of this form will correct for this error and such a correction may be applied in case the meter is used under conditions in which such a difference is of importance. For metabolism experiments in which the total volume is large compared with 180 cc., this error may be neglected. It is of about the order of magnitude of the error in reading the position of a spirometer bell.

The expired air is saturated with water vapor and is at a relatively high temperature. If it is, however, allowed to cool before being measured care must be taken that the meter is not warmer than the air for in passing through it will be warmed and no longer be vapor-saturated. To obviate this, on admitting the air to the meter, it may be passed through a water bottle slightly warmer than the meter. The temperature of the air whose volume is measured by the meter had best be taken by hanging a thermometer in the exit tube. The thermometer should reach its end-point quickly.

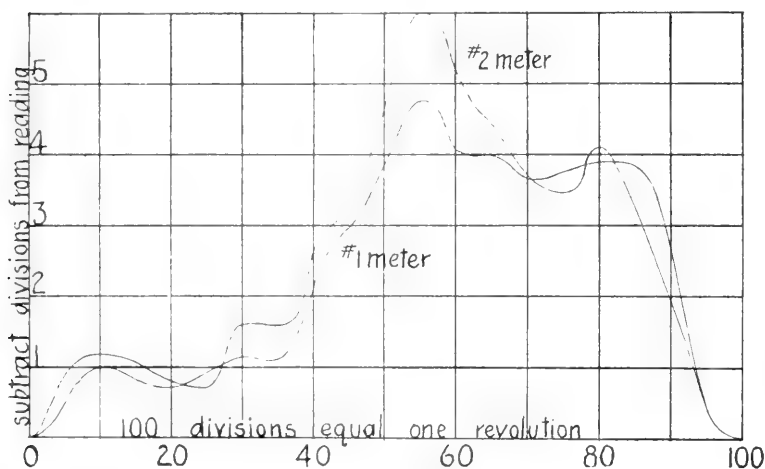


FIG. 2.

For the analysis of the expired air it is customary to use the portable gas analysis apparatus devised by Haldane (Fig. 3). The construction of this, as it is ordinarily obtained, is crude. It may be much improved by constructing a more adequate rack for the mercury leveling bulb and adding a similar one for the potash bulb. The various parts are usually connected by pieces of rubber tubing. These dry out and in any event are subject to leaks which are always suspected as possible sources of error. The principal glass parts may be blown in one piece so that there is only connecting rubber tubing below liquid levels. It is advisable to place a mercury trap between the burette and potash bulb, a feature which frequently saves much time. An additional improve-

ment is to place all the glass parts in a water bath instead of only the burette and its control. The apparatus is then less sensitive to currents of air producing temperature changes. Since it may be desirable to fill the gas burette to exactly 10 cc., it is necessary

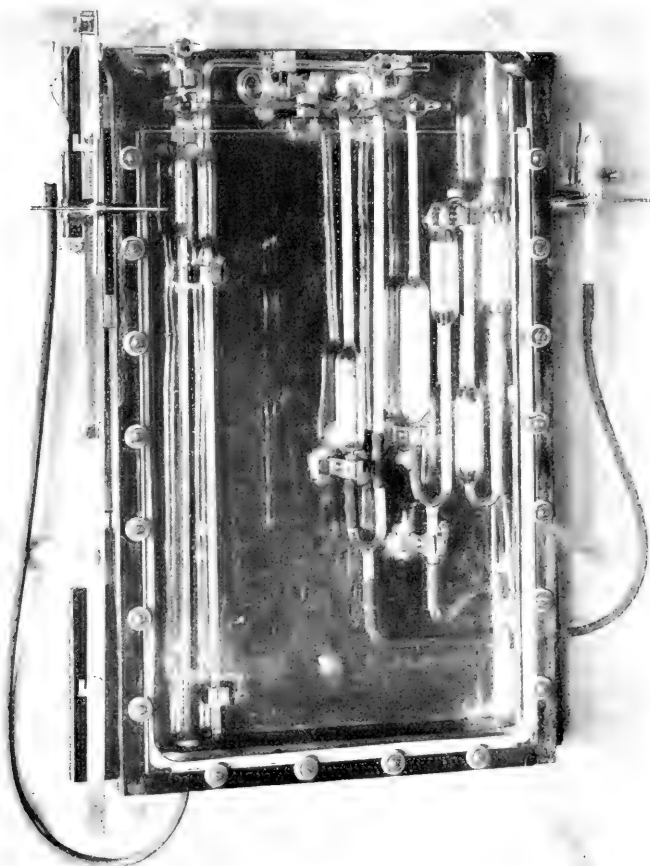


FIG. 3.

to have several graduations on the potash burette, about five at millimeter intervals, one of which may serve as an initial reading. The water bath may be constructed of a copper-lined box faced in front with plate glass, using rubber tubing as a washer between

the copper flange and the glass. The two racks and pinions may then be placed one at each side of the instrument and the connections to the leveling bulbs brought out through corks inserted in thimbles in the walls of the bath. These rather simple improvements do much to make the apparatus easier to work with.<sup>1</sup>

#### SUMMARY.

There is described a simple, accurate, and relatively inexpensive laboratory gas meter, being an adaptation of the common five light service meter.

Some improvements in the Haldane gas analysis apparatus are described.

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<sup>1</sup>The meter with dials attached and the modified Haldane apparatus may be obtained from the Arthur H. Thomas Co., Philadelphia.

## LIPASE STUDIES.

### I. THE HYDROLYSIS OF THE ESTERS OF SOME DICARBOXYLIC ACIDS BY THE LIPASE OF THE LIVER.

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(Received for publication, May 24, 1921.)

The hydrolysis of the esters of the dicarboxylic fatty acids, malonic and succinic, by lipase has not been the subject of careful study. Doyen and Morel (1) observed that serum lipase caused hydrolysis of diethyl succinate, but the results are difficult of interpretation from a quantitative standpoint. Kastle (2) stated that diethyl succinate was readily hydrolyzed by liver lipase, but cited no quantitative data as to the extent of hydrolysis. He also observed that the metallic salts of the monoethyl esters of the dicarboxylic acids, *e.g.* sodium ethyl succinate, were not split by the lipase of the liver. He considered that the inactivity of lipase toward this class of compounds was due to the fact that they are ionized and that lipase is unable to attack ionized compounds. Morel and Terroine (3) undertook a comparative study of the hydrolysis of the diethyl esters of malonic, succinic, glutaric, suberic, and sebacic acids by the pancreatic juice of the dog, and concluded that the ease of hydrolysis increased with the increase of molecular weight of the ester up to glutaric acid. The maximum hydrolyses obtained were 2.6 per cent for ethyl malonate and 12.9 per cent for ethyl succinate. It is difficult to interpret their results inasmuch as no very definite details as to the amounts of ester and enzyme used or the dilution employed are given. It would appear, however, that the concentration of ester was such that a considerable part of it must have remained in suspension or emulsion. Moreover, the time (6 to 7 hours in most experiments) was too short to allow the reaction to reach an equilibrium, especially with the high concentration of ester in

the reaction mixture. To obtain optimum conditions for the action of lipase, it is desirable that the concentration of the ester be such that complete solution is effected and that the dilution be sufficiently high to prevent inhibition of the action of the enzyme by the acid resulting from the hydrolysis of the ester.

In the experiments to be reported in the present paper the hydrolysis of diethyl malonate and diethyl succinate by the lipase of hog liver has been studied. Every attempt has been made to observe the optimum conditions for lipolytic activity in order to obtain the maximum hydrolysis possible. This has been effected by the use of dilute solutions (0.05 to 0.0125 N) and by frequent retitrations to neutralize the acidity developed which might inhibit the activity of the enzyme. The results obtained have confirmed the work of Kastle (2) on the stability of the metallic salts of the monoethyl esters of the dicarboxylic acids toward lipase. They indicate, however, that the hydrolysis of the diethyl esters studied can proceed only to the removal of one ethyl group and that the monoethyl esters as well as their metallic salts are stable toward lipase.

#### EXPERIMENTAL.

*Preparation of Lipase.*—The lipase was prepared from fresh hog liver by the use of glycerol as an extraction agent according to the method of Kanitz (4). The liver was obtained directly from the slaughter house and the extraction made within 1 hour after the death of the animal. After thorough admixture the extract was allowed to stand 2 months and then strained through cheese-cloth for use in the experiments. The use of a glycerol extract is advantageous since the glycerol not only furnishes a medium in which bacterial action is inhibited but also fails to extract fats and lipoids which by their autolysis might obscure the acidity developed by hydrolysis of the esters under investigation. Our observations are in agreement with the results of Simonds (5) who has reported that glycerol extracts of liver may retain their ester-splitting power unimpaired for some months.

*The Esters.*—The purity of the diethyl malonate and succinate used as substrates was checked by determinations of their boiling points and saponification values. The potassium ethyl malonate was a pure white crystalline compound. The preparation of the

acid ethyl malonate presented some difficulties. The procedure finally adopted was as follows. 4.25 gm. of pure potassium ethyl malonate were carefully weighed out and dissolved in a small volume of water, and the calculated amount of concentrated sulfuric acid was added. This was sufficient to convert the potassium salt to the monoethyl ester. After the addition of the acid the mixture was thoroughly extracted with twice its volume of ether and the extraction repeated twice. The combined ether extracts were then washed repeatedly with small amounts of water to remove traces of sulfates from the ether layer. After the evaporation of the ether at room temperature there remained a light-colored oily residue. This was carefully washed into a volumetric flask and made up to a liter with distilled water. No test for sulfates was obtained in this solution.

For the analysis of the monoethyl ester prepared as described 25 cc. of the solution were titrated with 0.09818 N sodium hydroxide using phenolphthalein as an indicator, and the saponification value of this neutralized solution determined in the usual manner. Results (averages of closely agreeing duplicate determinations) of the analysis of two different preparations are given.

25 cc. of solution required 4.10 cc. of 0.09818 N NaOH for the neutralization of the free acidity and 4.20 cc. of alkali for the subsequent saponification of the ethyl group.

25 cc. of the solution required 4.00 cc. of 0.09818 N NaOH for the neutralization of the free acidity and 4.20 cc. of alkali for subsequent saponification of the ethyl group.

These analytical results check satisfactorily with the theoretical for acid ethyl malonate, which would require that the alkali used for the neutralization of the free acid group should be equal to that used for the saponification of the ethyl group. It was computed also from these analyses that the normality of the solution of acid ethyl malonate was approximately 0.033 N.

*Determination of the Action of the Lipase on the Esters.*—All hydrolyses were carried out at room temperature. To 25 cc. of the ester (usually in 0.05 N concentration) was added 0.5 cc. of the strained glycerol extract of liver and after incubation for the desired period of time, the acidity developed was titrated with 0.09818 N sodium hydroxide with phenolphthalein as indicator.

The flasks were arranged in pairs and one pair titrated after 15 minutes, a second pair after 30 minutes, etc. Each pair of flasks was also retitrated at the intervals shown in the tables and the figures given represent the total volume of standard alkali required for neutralization. The figures presented in the tables are the averages of check determinations from which the blanks due to the acidity of the extract and of the esters have been subtracted. When ethyl propionate or butyrate has been used as substrate<sup>1</sup> it has been possible to obtain a hydrolysis of 85 to 90 per cent in 7 hours with the above procedure.

The significance of the tables will perhaps be made more evident by the following explanation. In Table I with 0.05 *N* ester, under the heading 1 hour, the figures 3.80, 3.30, 2.75, and 1.70 cc. are given. The figure 1.70 represents the average acidity developed in two flasks which were first titrated at the end of an hour. The figure 2.75 represents the average total acidity of two flasks which were first titrated at the end of a period of 45 minutes and again 15 minutes later at the end of an hour. Similarly 3.30 cc. of the standard alkali were required to neutralize the acidity in two flasks which were first titrated at the end of a period of 30 minutes, and then at 15 minute intervals at the end of periods of 45 and 60 minutes. It will be noted that the rate of hydrolysis is increased by neutralization of the acidity as it develops. Thus at the end of an hour in the case just discussed, the acidity produced was equivalent to 1.70 cc. of the standard alkali in the flasks not previously titrated, while the total acidities developed in those flasks in which 2, 3, and 4 titrations had been made during the same period were 2.75, 3.30, and 3.80 cc., respectively. This increased rate of hydrolysis continues up to the point of equilibrium and no further increase is then noted. Thus with 0.0125 *N* ester (Table I) the equilibrium point (1.35–1.40 cc.) is reached in 1 hour when the neutralization of acidity has been made at 15 minute intervals from the beginning of the experiment, and in 3 hours when no previous neutralization has occurred.

<sup>1</sup> Unpublished data.



TABLE I.

Comparative hydrolysis of 0.05, 0.025, 0.01666, and 0.0125 *N* solutions of diethyl succinate by lipase of hog liver, expressed in cc. of 0.09818 *N* NaOH required for neutralization of acid formed. 25 cc. portions of the ester solution were employed. For complete saponification 12.73, 6.36, 4.24, and 3.18 cc. of 0.09818 *N* NaOH, respectively, are required.

Time 15 min.	30 min.	45 min.	1 hr.	2 hrs.	3 hrs.	5 hrs.	7 hrs.	20 hrs.	Concentration of ester.
Standard NaOH required for neutralization of acidity.									
cc.	cc.	cc.	cc.	cc.	cc.	cc.	cc.	cc.	
0.70*	2.00	3.10	3.80	5.45	5.65	5.65	5.65	5.65	
	1.20	2.70	3.30	5.55	5.75	5.75	5.75	5.75	
		1.65	2.75	4.80	5.60	5.65	5.65	5.65	
			1.70	4.75	5.60	5.60	5.60	5.60	
				2.90	5.60	5.60	5.60	5.60	
					3.60	5.60	5.60	5.60	
						4.60	5.75	5.75	
							4.80	5.45	
								5.75	
									0.05 <i>N</i>
0.55	1.45	2.10	2.45	2.70	2.70	2.75	2.75	2.75	
	0.60	1.50	1.90	2.65	2.70	2.70	2.70	2.70	
		0.85	1.35	2.60	2.65	2.65	2.65	2.65	
			0.60	2.05	2.45	2.50	2.50	2.50	
				1.65	2.65	2.65	2.65	2.65	
					2.30	2.70	2.70	2.70	
						2.50	2.70	2.70	
							2.45	2.80	
								2.70	
									0.025 <i>N</i>
0.55	1.25	1.60	1.80	1.80	1.85	1.85	1.85		
	0.75	1.35	1.75	1.75	1.75	1.85	1.85		
		1.10	1.75	1.85	1.85	1.85	1.85		
			1.10	1.70	1.70	1.80	1.80		
				1.70	1.95	1.95	1.95		
					1.75	1.85	1.85		
						1.70	1.85		
							1.75		
									0.01666 <i>N</i>

\* In all the tables, the last figure in each vertical column represents the amount of alkali required for neutralization of the acidity developed during the period represented. Each figure in the horizontal column to the right of the first figure represents this amount of alkali plus the additional amounts of alkali required for retitration at the intervals indicated. For further explanation of this and succeeding tables see the text.

TABLE I—*Continued.*

Time 15 min.	30 min.	45 min.	1 hr.	2 hrs.	3 hrs.	5 hrs.	7 hrs.	20 hrs.	Concentration of ester.
cc.	cc.	cc.	cc.	cc.	cc.	cc.	cc.	cc.	
0.30	0.80	1.15	1.35	1.40	1.40	1.45	1.45		
	0.50	1.00	1.30	1.35	1.35	1.40	1.45		
		0.65	1.15	1.40	1.40	1.40	1.40		
			0.80	1.45	1.45	1.45	1.45		0.0125 N
				1.20	1.45	1.45	1.45		
					1.40	1.45	1.45		
						1.40	1.50		
							1.45		

## DISCUSSION.

In Table I are presented the data obtained from the hydrolysis of solutions of diethyl succinate in concentrations ranging from 0.05 to 0.0125 N by the lipase of the liver. It will be noted that regardless of the dilution of ester employed in no case was the hydrolysis greater than 50 per cent of the theoretical. Since with the esters of the monocarboxylic fatty acids (*e.g.*, ethyl propionate) hydrolysis was nearly complete under the same conditions this seemed to indicate either that at this stage equilibrium had been reached in the reaction or that the reaction was more nearly complete than appeared and that definite products were formed other than succinic acid and ethyl alcohol. Further consideration of the results showed that the percentage of hydrolysis in all the various dilutions closely approximated 90 per cent of the theoretical provided only one ethyl group was removed from the diethyl ester by the action of the lipase. The fact that equilibrium was reached at approximately the same point in each dilution would further indicate that a condition of equilibrium between diethyl succinate on the one hand and ethyl alcohol and succinic acid on the other at the point where about 45 per cent hydrolysis had occurred had not been reached. If the reaction were as incomplete as this would indicate, change in the dilution of the substrate should alter the station of equilibrium and the percentage hydrolysis increase with increasing dilution. Thus Taylor (6) has shown in his studies on the action of the lipase of the castor bean upon triacetin that under like conditions

a variation in the concentration of ester from 0.5 to 2.0 per cent resulted in a lowering of the percentage of ester hydrolyzed from 86 to 70. Kastle, Johnston, and Elvove (7) have also maintained that the more dilute the solution the greater the rate of lipolysis. In the present experiments the equilibrium corresponding to about 90 per cent hydrolysis of diethyl to monoethyl succinate was reached in the higher dilutions in 2 to 3 hours and remained unchanged thereafter.

The results of the experiments with diethyl malonate as substrate (Table II) are also in harmony with the theory that hydrolysis of the diethyl ester proceeds only to the removal of one

TABLE II.

Hydrolysis of 0.05 *N* diethyl malonate by lipase. 25 cc. of 0.05 *N* solution of ester were used. For the complete saponification of this amount of ester 12.73 cc. of 0.09818 *N* NaOH are required.

Time...15 min.	30 min.	45 min.	1 hr.	2 hrs.	3 hrs.	4 hrs.	5 hrs.	7 hrs.
Standard NaOH required for neutralization of acidity.								
cc.	cc.	cc.	cc.	cc.	cc.	cc.	cc.	cc.
0.65	1.25	2.05	3.05	3.85	5.05	5.65	5.95	6.05
	0.65	1.30	2.35	3.05	4.45	5.30	5.75	6.05
		1.00	2.15	2.95	4.50	5.30	5.80	6.00
			1.40	2.25	3.85	4.80	5.40	5.80
				1.60	3.30	4.30	5.10	5.80
					2.75	3.80	4.70	5.65
						3.30	4.35	5.60
							3.75	5.05
								4.00

ethyl group. The hydrolysis progressed rapidly to the point which corresponded to 85 to 90 per cent cleavage of one ethyl group and remained nearly constant thereafter.

In order to test the hypothesis that the second ethyl group of the diethyl ester could not be readily hydrolyzed by the lipase a study was made of the action of lipase on the acid ethyl malonate (Table III). No appreciable hydrolysis occurred in 24 hours. In order to determine whether this inactivity of the lipase was due to the inhibitory influence of the free acid group the action of lipase on potassium ethyl malonate was also investigated (Table IV). In confirmation of the work of Kastle (2) on sodium

ethyl succinate and other salts of monoethyl esters, no hydrolysis was observed. Inasmuch as both the acid ethyl ester and its potassium salt had been demonstrated to be stable toward the hydrolytic action of the lipase, a series of experiments was carried out to determine whether the acidity and the presence of potassium ions in the compound were the factors which inhibited or destroyed

TABLE III.

The action of lipase on acid ethyl malonate. (See text for the preparation of the acid tester.) 25 cc. portions were used. Initial acidity due to the acid group of ester plus the acidity of the glycerol extract is equivalent to 4.30 cc. of 0.09818 *N* NaOH. This blank has not been deducted.

Time 30 min.	1 hr.	3 hrs.	4 hrs.	5 hrs.	7 hrs.	24 hrs.
Standard NaOH required for neutralization of acidity.						
cc.	cc.	cc.	cc.	cc.	cc.	cc.
4.35	4.35	4.35	4.35	4.35	4.35	4.55
	4.30	4.30	4.30	4.30	4.30	4.65
		4.25	4.25	4.25	4.25	4.50
			4.30	4.35	4.35	4.55
				4.30	4.30	4.55
					4.30	4.50

TABLE IV.

The action of lipase on potassium ethyl malonate. 25 cc. of 0.05 *N* solution were used.

Time.....30 min.	1 hr.	2 hrs.	24 hrs.
Standard NaOH required for neutralization of acidity.			
cc.	cc.	cc.	cc.
0.00	0.00	0.00	0.10
	0.05	0.05	0.15
		0.05	0.15
			0.05

the lipase. Solutions were prepared containing the same concentration of the potassium and acid ethyl malonates as used in the previous experiments and ethyl propionate added to each. The action of lipase on these 0.05 *N* solutions of ethyl propionate in the presence of potassium and acid ethyl malonates is presented in Table V. The potassium salt of the ester exerted no demon-

strable influence on the course of the hydrolysis, the reaction proceeding at the usual rate. When acid ethyl malonate was present in the solution, the results were otherwise. No appreciable splitting of the easily hydrolyzed ethyl propionate occurred in 4 hours and little in 24. These experiments would indicate that the acidity of the monoethyl ester of the dicarboxylic acids studied may be one factor which inhibits the action of lipase on this type of compounds.

TABLE V.

The action of lipase on 0.05 *N* solutions of ethyl propionate in the presence of acid ethyl malonate and potassium ethyl malonate. 25 cc. portions were used. 12.73 cc. of 0.09818 *N* NaOH are required for complete saponification of this amount of ethyl propionate.

0.05 <i>N</i> ethyl propionate and potassium ethyl malonate.				
Time. .2 hrs.	4 hrs.	12 hrs.	17 hrs.	24 hrs.
Standard NaOH required for neutralization of acidity.				
cc.	cc.	cc.	cc.	cc.
3.30	6.25		7.80	
	4.00		8.15	
		6.95	9.55	
0.05 <i>N</i> ethyl propionate and acid ethyl malonate.*				
0.45	2.35			
	0.50			
				2.30

\*The blank due to initial acidity of acid ethyl malonate has been subtracted.

Since all the experimental evidence thus far obtained indicated that the lipase of the liver could split the diethyl esters of the series under investigation only to the monoethyl esters, an attempt was made to isolate and identify the monoethyl derivative among the products of the lipolytic action. 3.75 cc. of ethyl malonate were added to water (100 cc.) with 5 cc. of the glycerol extract, and hydrolysis was allowed to proceed with frequent neutralization of the acidity by 0.09818 *N* sodium hydroxide, until the acidity developed over a period of several hours became almost negligible. For complete hydrolysis of this amount of ester, 506

cc. of the standard alkali should have been required, but it was found that even with frequent retitrations the reaction reached an equilibrium when about 240 cc. of alkali had been added, a figure which is in rather striking confirmation of the experimental results obtained previously with smaller amounts of ester. The reaction mixture was then filtered and carefully extracted three times with ether. This should have removed any unchanged diethyl malonate which would have been present in considerable amount if malonic acid and ethyl alcohol had been the products and the reaction had reached an equilibrium at a hydrolysis of less than 50 per cent as indicated by the titration figures. If on the other hand, the products formed were acid ethyl malonate and ethyl alcohol, the amount of unchanged diethyl malonate should have been very slight. The ether extract was carefully evaporated at room temperature, and the residue dissolved in water. Aliquots of this solution contained no material which could be saponified either by alkali or by the glycerol extract of hog liver. This furnished evidence that no significant amounts of diethyl malonate remained after hydrolysis by lipase.

The solution which remained after the ether extraction was acidified with sulfuric acid and repeatedly extracted with ether. The combined ether extracts were washed several times with water to remove traces of sulfates and sulfuric acid, and the ether removed by evaporation at room temperature. The oily residue of a light yellow color was dissolved in water and made up to 200 cc. No sulfates were detected in this solution. Quantitative tests were carried out on aliquots in the manner similar to that outlined in another part of this paper for the analysis of the acid ethyl malonate prepared from potassium ethyl malonate. If the chief product of the reaction had been malonic acid, then no saponifiable material should have been present after neutralization of the free acid. If ethyl acid malonate had been formed, however, the alkali required for neutralization of the free acid and that for the subsequent saponification of the ethyl group should have been the same. Analysis showed that 25 cc. of the preparation described required 16.30 cc. of 0.09818  $N$  sodium hydroxide for neutralization of the free acidity and 17.43 cc. for the subsequent saponification of the neutralized aliquot. Analyses of the products of another experiment carried out under similar

conditions showed titrations of 16.25 and 18.17 cc., respectively. When the possible sources of error in an experiment of this sort are considered, these results may be considered satisfactory and to offer further proof in support of the theory that the monoethyl ester is the main product of the hydrolysis of the diethyl esters of succinic and malonic acids by the lipase of hog liver and that the monoethyl ester is hydrolyzed with difficulty, if at all. Further studies to determine whether lipases from other sources behave similarly are in progress.

#### SUMMARY.

On the basis of the acidity developed when the lipase of hog liver was allowed to act upon the diethyl esters of succinic and malonic acids, it is considered that the reaction proceeded to an equilibrium which corresponded to the removal of one ethyl group from the diethyl esters. A substance was obtained from the products of the reaction between diethyl malonate and lipase which gave on analysis figures which were in good agreement with those required for monoethyl malonate. Lipase of hog liver was not able to effect the cleavage of monoethyl malonate or potassium ethyl malonate. Ethyl propionate was hydrolyzed by lipase in the presence of the potassium salt of monoethyl malonate, but not in the presence of the monoethyl ester itself.

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## STUDIES ON EXPERIMENTAL RICKETS.

### VIII. THE PRODUCTION OF RICKETS BY DIETS LOW IN PHOSPHORUS AND FAT-SOLUBLE A.

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PLATES 4 TO 7.

(Received for publication, June 20, 1921.)

#### INTRODUCTION.

In a brief preliminary report<sup>1</sup> we described a faulty ration and its effects on the skeleton of the growing rat, which corresponded in all essential details to rickets as that disease manifests itself in the skeleton of the human being. The faulty ration in question, No. 3127, was deficient in phosphorus and fat-soluble A.<sup>2</sup> In other respects it was, apparently, satisfactorily constituted. It contained protein of good quality and adequate in amount. The calcium was furnished in the form of the carbonate in a proportion (2 per cent of the total ration) which was essentially the amount necessary for the best promotion of growth, longevity, and reproduction. We took pains to point out that, when the

<sup>1</sup>Read before the American Pediatric Society, Swampscott, Mass., June 3, 1921.

<sup>2</sup>We are at present unwilling to commit ourselves to the view that the antixerophthalmic factor and the factor concerned in the ossification and growth of the skeleton are distinct, though we are prepared to acknowledge that such may be the case. We, therefore, use fat-soluble A to describe both the antixerophthalmic and antirachitic factors, assuming that the latter has a separate existence.

content of phosphorus in the faulty ration was raised through the substitution of 2.5 per cent  $\text{CaHPO}_4$  for the 2 per cent  $\text{CaCO}_3$ , osteoporosis and not rickets developed. We also called attention to the fact that, when a deficiency in calcium as well as in phosphorus was created through the omission of the  $\text{CaCO}_3$  from the faulty ration, a pathological condition developed in the skeleton which was entirely distinct from rickets. We showed further that, when cod liver oil was added to the faulty ration in an amount equal to 2 per cent by weight of the ration, the pathological condition which developed in the skeleton was osteoporosis and had no resemblance whatsoever to rickets. We believe the results obtained in the experiments cited to be of considerable significance. In the first place, the experiments showed that it was possible to produce a pathological condition in the rat unquestionably similar to the rickets of the human being through the diet alone. In the second place, they showed that rickets could be induced by means of a ration, the faults of which were clearly defined and sharply limited; *viz.*, deficiencies in phosphorus and fat-soluble A. In the third place, they indicate that deficiency of phosphorus in the ration insufficiently supplied with fat-soluble A would give rise to rickets only when calcium was present in a ratio considerably higher than the calcium-phosphate ratio which is optimal for ossification. The experiments indicated, therefore, that the development of rickets in this instance depended on the existence in the faulty ration of (1) a specific disproportion in the calcium-phosphate ratio, the phosphorus being low, the calcium, relatively speaking, high, and (2) an insufficiency of an organic substance contained in cod liver oil having a profound influence on the calcification of cartilage and the ossification of bone.

In the present paper we wish to describe further the composition of the faulty ration which produced the rachitic lesions above referred to, and to present in detail the anatomical evidence on which the statement of the production of rickets was based. We also wish to describe two other similarly constituted diets, one being the diet just referred to only slightly modified, and their effects on the growth and ossification of the skeleton in the rat.

## Ration 3127.

	per cent
Rolled oats.....	40.0
Gelatin.....	10.0
Wheat gluten.....	7.0
NaCl.....	1.0
KCl.....	1.0
CaCO <sub>3</sub> .....	2.0
Dextrin.....	39.0

This mixture was markedly deficient in phosphorus and in fat-soluble A (antixerophthalmic substance) but was otherwise well constituted. Its proteins were abundant and of good quality; its content of calcium not far from the optimum. So far as we can judge from experimental data available, the content of other inorganic constituents was satisfactory. While it did not furnish much above the actual requirement of the antineuritic substance, this factor could not be regarded as influencing in any way the well being of the animals.

TABLE I.

## Data Concerning Rats of Lot 3127.

No. of rat.	Age when put on diet.	Weight when put on diet.	Days on diet.	Age at death.	Xerophthalmia.	Sex.	Weight.
	days	gm.		days			gm.
642	25	41*	24	49	+	♀	41
688	25	41	35	60	+	♂	48
689	25	41	35	60	+	♀	46
690	25	41	35	60	+	♂	46
708	25	41	39	64	+	♂	48

\*The entire litter of rats composing Lot 3127 was weighed together. Each recorded weight in the column does not represent the exact weight of the individual but one-sixth the total weight of the litter. The sixth rat was partly eaten after being on the diet 35 days.

Ration 3133 was closely similar to Ration 3127 except that the former contained 0.5 per cent of butter fat in place of an equivalent amount of dextrin. This supplied a small amount of the fat-soluble A (antixerophthalmic substance) but not sufficient to entirely protect the animals against ophthalmia. This addition of butter fat was not sufficient to enable them to grow, although more liberal amounts would have done so. We know from other

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experiments, however, that they could not have been normal or have developed normal bones even with very liberal amounts of butter fat. The 0.5 per cent of butter fat added to their diet extended the lives of the animals to some extent.

TABLE II.  
*Data Concerning Rats of Lot 3133.*

No. of rat.	Age when put on diet.	Weight when put on diet.	Days on diet.	Age at death.	Xeroph- thalmia.	Sex.	Weight.
	<i>days</i>	<i>gm.</i>		<i>days</i>			<i>gm.</i>
590	16	29*	35	51		♀	35
639	16	29	43	59		♀	27
706	16	29	59	75		♂	67
717	16	29	61	77		♀	45
811	16	29	91	107		♂	60
860	16	29	98	114		♂	39

\*The entire litter of rats composing Lot 3133 was weighed together. Each recorded weight in the column does not represent the exact weight of the individual but one-eighth the total weight of the litter. Two rats disappeared from the cage; they were probably eaten.

### *Ration 3133.*

	<i>per cent</i>
Rolled oats.....	40.0
Gelatin.....	10.0
Wheat gluten.....	7.0
NaCl.....	1.0
KCl.....	1.0
Dextrin.....	38.5
CaCO <sub>3</sub> .....	2.0
Butter fat.....	0.5

### *Ration 3143.*

	<i>per cent</i>
Wheat.....	33.0
Maize.....	33.0
Gelatin.....	15.0
Wheat gluten.....	15.0
NaCl.....	1.0
CaCO <sub>3</sub> .....	3.0

This diet contains nearly twice the optimal content of calcium and is decidedly below the optimum in its content of phosphorus and of fat-soluble A. Otherwise it is well constituted. On this

food mixture young rats grow somewhat more than on the other diets described in the paper. They do not develop xerophthalmia but become very deformed and die early.

When placed upon Ration 3127 the young rat lives for not more than 5 to 8 weeks. The growth momentum derived from the normal diet results in a preliminary gain. This soon ceases, however. The weight then remains stationary and declines toward the end of the experiment. The rats of Lot 3127 were 25 days old when confined to the faulty ration, and were killed after periods varying from 24 to 39 days.

TABLE III.  
*Data Concerning Rats of Lot 3143.*

No. of rat.	Age when put on diet.	Weight when put on diet.	Days on diet.	Age at death.	Xerophthalmia.	Sex.	Weight.
	<i>days</i>	<i>gm.</i>		<i>days</i>			<i>gm.</i>
571	50	64	50	100		♂	81
657	50	65	63	113		♀	82
658	50	67	63	113		♀	82
831	45	76	43	88		♂	62
879	40	60	60	100		♂	65
914	40	55	65	105		♂	68
915	40	53	65	105		♂	75
918	40	60	76	116		♀	60
919	28	56	69	97		♀	63

At autopsy the bodies were small and greatly emaciated. Almost no fat was present. The eyes were inflamed. The incisor teeth were brittle. The thorax was flattened at the sides, and in one or two animals was hollowed slightly along the line of junction of the cartilages and the ribs. In none of the animals, however, was it greatly deformed. The costochondral junctions themselves formed fusiform enlargements, most evident on the internal surface of the thorax. The enlargements were thrown into especial prominence if the thoracic wall was bent inwards. Fractures of the shaft of the ribs with callous formation situated usually not far behind the costochondral junctions were present in three of the animals. The lower end of the radius and ulna and the adjacent ends of the femur and tibia formed enlargements similar to those seen in the rachitis of human beings. When stripped of their

muscles the ends of all the long bones were found to be enlarged. Fractures of the long bones of the extremities were not noted. The strength of the tibia and femur was greatly reduced and both these bones and the ribs offered little resistance when cut. Examination of the cut surface of the femur or tibia showed that the junction of cartilage and shaft was not clearly defined and that below the cartilage there lay a pale yellow zone of not very great depth which proved when examined under the microscope to be a transitional zone containing a mixture of elements derived from cartilage and shaft. Examination of the cut surface further showed the cortex to be extremely thin and the bone marrow to have approximately normal color.

The following changes were noted when the bones were examined under the microscope.<sup>3</sup> The epiphyseal cartilage separating the nucleus of ossification from the shaft was in most places broader than in the healthy animal and at several points was continued in irregular blue-staining prolongations toward or actually to the shaft. Its diaphyseal border did not end abruptly on coming in contact with the elements derived from the diaphysis but seemed to undergo a gradual transition into the latter. The zone of undifferentiated cartilage was represented by few cells. Almost at their beginning, close to the nucleus of ossification, the cells of the epiphyseal cartilage became arranged in columns or fascicles separated by rather more matrix than is seen in the bones of young rats on normal diets. Near their points of origin the cells composing the columns were flattened as if compressed in the long direction of the shaft and lay close to each other, but at a short distance from their points of origin became oval, round, or cuboidal. As soon as the cells of the epiphyseal cartilage underwent the expansive change just referred to, the intercellular substance which separated the cell groups became greatly diminished, so that the columns appeared to lie close to each other. Coincidentally, the cells seemed to lose to a certain degree their affinity for hematoxy-

<sup>3</sup>The technique employed in these experiments was as follows: The bones were hardened in formaldehyde. They were cut after embedment in celloidin in sections 15 to 25 mikra thick and stained with hematoxylin and eosin. Control preparations were made with the freezing microtome and stained with hematoxylin and also silver nitrate after the method of von Kossa.

lin with the result that both nucleus and cell body appeared more pale. The cell membrane appeared to be unusually thick. In general the nuclei were large, round, and well preserved. The columnar arrangement of cells was at some points maintained throughout the breadth of the cartilage, but at others became irregular or entirely lost as the metaphysis was approached, so that the cells seemed to be irregularly jumbled together. Wherever the cartilage cells came in proximity to the marrow elements they lost their blue color and were pale yellow. In many places the cartilage after losing its staining reaction ended abruptly in contact with the blood vessels derived from the marrow. At other places, however, the cartilage was continuous with the cartilage which formed the bulk of the transitional zone between cartilage and shaft, the rachitic metaphysis (Fig. 3).

The metaphysis in which the elements derived from cartilage and shaft were blended in an irregular manner was not deep. Indeed in comparison with the metaphysis in the bones of the rats on Rations 3133 and 3143, it was exceedingly shallow. It was composed of the irregular prolongations of cartilage from the main mass which retained their blue stain, and of quantities of cartilage cells which had lost their staining reaction to hematoxylin entirely and were in all stages of transition into osteoid. There were osteoid trabeculae derived largely from cartilage and having a waxy appearance. The remains of calcified intercellular substance staining deep blue and having a configuration like the cross-section of a honeycomb were formed in the metaphysis. The detailed description of the various elements composing the metaphysis will be found in the description of the histological findings of the rat on Ration 3133 (Fig. 4).

The cortex of the shaft near the metaphysis was broad and composed of a thick interlacing network of trabeculae having for the most part central cores of calcified material and borders of osteoid. As the middle of the bones was approached, however, the cortex became thin. The trabeculae had a central core of calcified material but peripheral portions composed of osteoid. The osteoid zones were fairly broad. In most of them a fibrillary arrangement could be made out. The bone corpuscles of the calcified portion were small and those in the osteoid borders appeared especially small and far separated from each other. They pre-

sented a variety of shapes. Some were round, some spindle-shaped, some irregular. Moreover, they were very unevenly distributed. Some resorptive phenomena could be recognized in the calcified portions of the trabeculae. The marrow appeared normal. The nucleus of ossification showed changes both in its cartilaginous elements and in the trabeculae exactly analogous to those described in the epiphyseal cartilage, and in the shaft.

The rats on Ration 3133 were younger (only 16 days old) when placed upon the faulty mixture than the rats of the lot on Ration 3127, and were kept on the faulty diet for longer periods of time, on the average 63 days. The shortest period was 35 days and the longest 98 days. The rats scarcely more than maintained their initial weight on Ration 3133. They were small and poorly nourished. The eyes of most of them were inflamed. The thorax was considerably deformed. The costochondral junction was considerably enlarged. In the majority of the animals they were displaced inwards and were greatly distorted. Fractures of the ribs were present with well marked callous formation. The knees, wrists, and ankles were considerably enlarged, as were the ends of all the long bones. The femurs and tibias cut with greatly diminished resistance. On section the enlargement of the ends of the long bones was especially evident and seemed to correspond to the cartilage and to a white zone between the cartilage and bone marrow which on microscopic examination proved to be the rachitic transitional zone or metaphysis. The line of junction between the cartilage and shaft was indistinct. The shafts of the long bones were not enlarged. The spleen was not increased in size in the majority of the rats but was enlarged in some.

The microscopic changes in the femurs and the tibias of the rats on this diet resembled those described in the bones of the rats on Ration 3127, but were considerably further advanced. The epiphyseal cartilage was irregularly increased in depth and seemed to merge with the metaphysis in a manner presently to be described. The breadth of the metaphysis was almost if not quite equal to the breadth of the epiphyseal cartilage. The shaft of the bone was broad where it bordered on the metaphysis but rapidly contracted so that the shaft of the bone as a whole did not seem to be enlarged (Fig. 2).



The undifferentiated cartilage was represented by only a few scattered cells or cell clusters. Almost at their beginning the cartilage cells became arranged in short columns or rather groups of columns. These were more widely separated from each other by matrix than in the healthy animal; that is, the amount of matrix seemed to be considerably in excess of that found in the rat under normal conditions. The columns or bundles of cartilage cells just referred to took their origin at somewhat different levels. They were bullet-shaped and the ends of the columns directed toward the nucleus of ossification were for the most part pointed. The cartilage cells composing them were flat, the long dimension running from side to side. Some of them had the same thickness throughout, others were spindle- or wedge-shaped. Many of them were curved like crescents, the concave side of the crescent opening diaphysealwards. The nuclei were in some instances centrally placed, in others at the side. The nuclei were long and thin, partaking of the shape of the cell, and took a very dark stain with hematoxylin. In some cells no cytoplasm could be seen; in all, the cytoplasm if present was scant and took a paler blue stain than the cell capsule or nucleus. The cell capsule was not clearly defined and could not be easily separated from the adjacent cell capsules or surrounding matrix. In most of the bundles the arrangement of the cartilage cells was quite irregular. The cells lay one upon the other, but not in continuous columns. One row of cartilage cells would appear to interdigitate with another or a third column would seem to be interpolated between two rows of cartilage cells. The matrix in the part of this portion of the proliferative cartilage near the nucleus of ossification stained blue with hematoxylin but as the hypertrophic zone of the cartilage was approached it began to lose its blue color and appeared yellow.

As the metaphysis was approached the cells of the cartilage underwent a remarkable change. The cells forming the bundles suddenly expanded assuming globular, oval, or cuboidal shapes and at the same time began to lose their power to take the blue stain of the hematoxylin and soon ceased to take it altogether. The nuclei shared in the expansion of the cells, becoming large, round, and vesicular, and took a pale blue stain. The matrix between the cell bundles which had lost its blue color earlier

than the cells themselves was stained a pale yellow after hematoxylin and eosin. As the cells increased in size, the broad zones of matrix which separated them became somewhat decreased in amount with the result that the columns lay closer to each other. In many places, however, the broad zone of matrix which separated the bundles of flat cells above continued to exist between the columns of swollen cells nearer the diaphysis. As the cartilage cells underwent the sudden enlargement and alteration in staining reaction their columnar arrangement tended to become more definite. In certain places in which the cartilage was prolonged in solid masses into the metaphysis, the cells tended to retain their affinity for hematoxylin. The morphology of the cells in these irregular prolongations will be discussed later. *Calcification of the cartilage was entirely wanting in all the animals on this diet.*

The metaphysis varied considerably in its depth, and in its general character. In the majority of the animals it was deep. In one rat, however, which weighed only 25 gm. when killed, it was shallow. In all the animals it was made up of the various elements derived from cartilage and shaft which usually compose the metaphysis of a rachitic bone intermingled in an irregular manner. There were the irregular prolongations of the epiphyseal cartilage just referred to which retained their staining reactions to hematoxylin. There was cartilage which had lost its staining reaction altogether and seemed to be in various stages of transformation into osteoid. Many blood vessels were present, filled with red blood cells and surrounded by marrow elements. There were remnants of calcified matrix and in some rats considerable amounts of fibrous tissue (Fig. 5).

In all of the rats by far the greater part of the metaphysis was composed of the cartilage described as having lost its staining reactions entirely in the process of transformation into osteoid. This cartilage appeared in the sections lightly colored as cream color or pale yellow with eosin. It had exactly the same staining reaction as the osteoid borders of the trabeculae. These cartilage cells for the most part looked swollen and presented a great variation in size, shape, and general morphology and composed the main bulk of the metaphysis. Many were large, and round, oval, or cuboidal in shape. Some were pear-shaped and some

flattened. The nuclei were for the most part large and round and stained pale blue but in some the nuclei were small and deep blue or pycnotic. The cell bodies were yellow like the surrounding matrix or were actually colorless. The capsules appeared to be exceedingly thin. They were colorless in the case of many of the cells and could be distinguished only with reduced light as a transparent circle surrounding the cell. In the case of other cells the capsules retained some of the blue stain. The cartilage cells which had thus lost their staining reaction were anything but uniform. A row of globular cells like those just described might be continuous with a group of small cells, the smallest perhaps no larger than osteoblasts with correspondingly small nuclei staining deeply with hematoxylin, or they might be continuous with cells having a flat contour like those described in the cell bundles near the epiphyseal nucleus. Cells could be found which seemed to be fading away into a substance indistinguishable from the surrounding matrix. No nuclei seemed to be present in them, or the nuclei were so faintly stained as to be scarcely visible and the existence of the cells could be recognized only by the faint outline of the cell body visible under reduced illumination. In general, the cells in the metaphysis near the cartilage tended to be disposed in columns which were continuous with the columns of the cartilage proper. On the diaphyseal side of the metaphysis, however, the columnar arrangement had very largely disappeared. The large globular forms of cartilage cell were more numerous near the epiphyseal cartilage. The small forms were especially common on the diaphyseal side. The degenerating forms, also much more frequent on the diaphyseal side, were especially numerous in the immediate neighborhood of the blood vessels. Some of the blood vessels seemed to be surrounded by matrix exactly like the osteoid covering the trabeculae in its staining properties and almost devoid of nuclei. The amount of matrix in which the cartilage cells lay was present in much greater quantity on the diaphyseal side of the metaphysis than on the side of the epiphyseal cartilage (Fig. 7).

The cells of the cartilage which retained their staining property were for the most part those composing solid prolongations from the main mass of epiphyseal cartilage. Blood vessels did not penetrate them. The retention of their normal staining proper-

ties and a more nearly normal morphology may have been due to the fact that they happened to have been protected from the influences of the diaphyseal circulation. The cells composing these irregular prolongations, however, showed considerable morphological variation. Near the epiphyseal cartilage they were frequently indistinguishable from the large globular cells close to the bundles of flattened cells. As the diaphysis was approached, however, the cells perhaps assumed a flat shape and appearance exactly comparable to the cells forming the bundles at the beginning of the epiphyseal cartilage. Nearer the diaphysis they might again become large and round and finally they might again become flat or show evidence of partial calcification or break up into small groups composed of cells globular in form and much reduced in size, with clearly defined rather thick blue staining cell capsules and separated by an abundant matrix which had lost its staining reactions to hematoxylin altogether. Blood vessels penetrated the metaphysis from the shaft, and were found at the level at which the sudden transition in the cartilage above described took place; *i.e.*, to the level at which the cartilage cells suddenly became swollen and lost their staining properties. This fact would seem to indicate that the changes in the cartilage might be initiated as the result of circulatory influences. Blood vessels of all sizes could be seen ramifying in an irregular manner through the diaphysis. For the most part, however, they were not large. The large vessels terminating in huge tufts so frequently seen in the bones of rachitic human beings were not present. The blood vessels were filled with red blood cells and were surrounded by marrow elements. In general, it would seem that they penetrated the cartilage rather by insinuating themselves along the intercellular ground substance separating the columns than by destruction of the cartilage cells themselves. Occasionally, however, places could be found in which the red blood cells were present within the cartilage cell capsule. Evidently under the abnormal conditions induced by the diet the cartilage cells could be destroyed by the vascular elements, but in general tended to persist, undergoing a slow degeneration or change into hyaline substance or possibly a change of a metaplastic nature into osteoid or connective tissue. In general, the blood vessels had a delicate endothelial lining but groups of red blood

cells could be found lying in spaces in the metaphysis without evidence of surrounding endothelium. In general, the metaphysis did not appear to be disrupted by the vascular elements to the extent frequently seen in the bones of rachitic human beings.

Though in none of the bones of the animals on this diet was there calcium deposition in the cartilage, in almost all there were irregular calcium deposits at one point or another in the metaphysis. In one animal a zone of calcium deposition extended completely across the metaphysis, bisecting it. The calcium deposits were delicate and for the most part completely surrounded the cartilage cells, having in sections an appearance like that seen in a cross-section of a honeycomb. The cartilage cells in the metaphysis encased in calcified matrix showed morphological changes and loss of staining reactions comparable to those in evidence in the cartilage of the metaphysis elsewhere. In some of the interstices of the calcified matrix red blood cells and other marrow elements could be seen.

In one or two of the rats there was considerable connective tissue formation exactly similar to the fibrous tissue commonly seen in the rickets of human beings. As the shaft was approached the numbers of the blood vessels increased and the numbers of cells of diaphyseal origin became more numerous. Some of them bordering the blood vessels had the morphology of osteoblasts, others of connective tissue cells. Trabeculae devoid of any calcified matter but containing instead cores of cartilage cells which varied greatly in size and were in various stages of transformation or degeneration into osteoid were seen in the shaftward portion of the metaphysis. The junction of the diaphysis and metaphysis in most of the rats was marked by calcified intercellular substance indicating the level in the growing end of bone at which the effect of the faulty diet first became manifest.

The cortex was thin and the trabeculae were covered with osteoid. The cells of the osteoid investments were for the most part elongated, resembling connective tissue cells, and were widely separated from each other. The osteoid had a fibrillar arrangement. In some of the animals there were large osteoid formations on one side of the bone or the other under the periosteum. Few evidences of resorptive activity could be found. The marrow elements appeared to be essentially normal (Fig. 6).

In experiments with the rats of Lot 3143 the normal diet was stopped and the faulty ration substituted at ages ranging from 28 to 50 days. The animals were allowed to live on the faulty ration for from 43 to 76 days. When placed upon the faulty ration they continued to gain slowly or remained stationary in weight. They never developed inflammation of the eyes (xerophthalmia) on account of the presence of sufficient protective substance in the wheat and maize of the diet. If this diet is continued over a sufficient length of time the animals die. Just before the end of their lives rats on this ration show a definite loss of control of the posterior extremities which results in a tottering gait. The trouble is progressive and finally results in a complete paralysis of the hind legs. This condition will be fully discussed in another communication.

At autopsy they were small and emaciated. The incisor teeth were loose and so brittle as to be easily fractured. The thorax was greatly deformed. Its anteroposterior diameter was greatly increased as compared with its lateral diameter and the lower part of the sternum projected forward as in the pigeon breast deformity of human beings. At the line of the costochondral junctions there were deep grooves. When the thorax was opened and the interior examined the deformity was enormous. The costochondral junctions and shafts of the ribs met at acute angles, the apices of which pointed toward the vertebral column and were not far from it. The costochondral junctions themselves were exceedingly large and some of those of the lower ribs were twisted into the most bizarre shapes. The posterior epiphyseo-diaphyseal junctions were enlarged and appeared like so many white beads on either side of the spinal column. Many fractures of the shafts of the ribs were present, marked by large white callous formations. The spinal column itself was bent in various curvatures. The wrists, knees, and ankles were greatly enlarged, as indeed were the ends of all the long bones. Even the scapulae were deformed. Fractures of the tibia marked by angular deformity and huge callous formation were present in some of the rats at the junction of the upper third with the lower two-thirds.

The description of the microscopic changes in the bones of the animals on this diet corresponds in its main features to the description already given of the microscopic changes in the rats on

Ration 3133. We shall limit the description of the microscopic changes, therefore, to a few observations. The enlargement of the ends of the long bones was due to an increased depth and probably also to an increased breadth in the epiphyseal cartilage and to the presence of an enormous metaphysis. The width of the metaphysis was equal to that of the epiphyseal cartilage; its depth was many times greater. The groups of flattened cartilage cells constituting the columnar zone of the cartilage were very widely separated from each other by hyaline matrix. It was impossible not to think that the amount of hyaline matrix throughout the cartilage was greatly increased. The hyaline matrix early lost its power to take the hematoxylin stain. As soon as the cartilage cells had undergone the expansive change fully described in the case of the rats on Ration 3133 they rapidly lost their normal staining properties and underwent the changes of a metaplastic and degenerative character already described. By far the greater part of the metaphysis was composed of cartilage or derivatives from the cartilage. The osteoid trabeculae of the metaphysis in the main had a cartilaginous basis; that is, in almost all of them cartilage cells in various stages of degeneration or metaplasia could be identified. The cartilage in every animal of the group was entirely free from lime salt deposits. Some irregular lime salt deposition was present in the metaphysis of certain of the animals. The blood vessels which ramified through the metaphysis were in the main small. The cortex was thin. It was composed of bone-containing scanty central cores of calcified material and with broad osteoid borders. The breadth of the osteoid borders was extreme. The bone corpuscles found in them were widely separated from each other by quantities of yellow osteoid material. The number of cells per unit of area was consequently greatly diminished. The nuclei were long, some were spindle-shaped. They resembled the nuclei of connective tissue cells much more closely than those of bone corpuscles. A fibrillar arrangement of the osteoid could be made out. Few osteoblasts or at least cells which could be identified as such could be found lining the osteoid trabeculae. The evidences of resorption of the calcified portions of the trabeculae were slight. The bone marrow appeared to be essentially normal (Fig. 1).

Osteoid production was extreme in the epiphyseal nuclei of ossification. The large calluses which were formed about healing fractures were composed entirely of osteoid tissue.

#### DISCUSSION.

The faulty rations, Nos. 3127 and 3133, containing 2 per cent added calcium carbonate, gave rise to pathological conditions in the skeleton essentially identical with those found in the human subjects of rickets. The rachitic lesions in the group of rats on Ration 3133 were more advanced than those in the group of animals on Ration 3127, presumably on account of the presence in Ration 3133 of 0.5 per cent of butter fat. This small quantity of butter fat, while insufficient to prevent the development of xerophthalmia, was sufficient to delay its advent and to diminish its rate of progress. It increased the duration of life and made possible, presumably, increased growth of the skeleton. The small amount of butter fat in Ration 3133 probably intensified the rickets-producing properties of the diet through its stimulating effect on growth and its favorable influence on the duration of life. We have already called attention to the fact that butter fat as compared with cod liver oil is exceedingly low in its rickets-inhibiting properties.<sup>4</sup>

Ration 3143 was derived in part from wheat and maize, which contained a certain amount of antixerophthalmic substance (more than was present in Ration 3133, which contained 0.5 per cent butter fat). Ration 3143 further differed from Rations 3127 and 3133 in having a higher calcium-phosphate ratio than either. It gave rise to the most extreme degree of rickets. The osteoid production and the metaplastic and degenerative changes in the cartilage exceeded anything ever seen in the bones of rachitic human beings. We have repeatedly observed that, if calcium carbonate in large quantities (3 to 6 per cent of the total ration) is added to a ration insufficiently supplied with the organic factor and only slightly or not at all deficient in its content of phosphorus, most pronounced changes occur at the growing ends of the long bones. The cartilage undergoes the degenerative and metaplastic

<sup>4</sup> McCollum, E. V., Simmonds, N., Shipley, P. G., and Park, E. A., *Proc. Soc. Exp. Biol. and Med.*, 1920-21, xviii.



changes already described, and fails to take up calcium phosphate with any regularity, or to take it up at all. The trabeculae near the end of the shaft become surrounded with enormous quantities of osteoid and great quantities of osteoid trabeculae largely derived from the cartilage develop between the cartilage and the shaft. The exaggerated character of the rachitic lesion brought about by Ration 3143 is explicable on the ground that the phosphate is extremely low and the calcium high (3 per cent of the total ration in the form of calcium carbonate), so that the calcium-phosphate ratio was exceedingly high. The quantity of the antixerophthalmic substance contained in Ration 3143 was sufficient to prevent effectually the development of xerophthalmia. The quantity of the antixerophthalmic substance contained in the diet, however, was insufficient to exert, or incapable of exerting, any visible inhibitory influence on the development of the rachitic lesions in the skeleton. Indeed, it seems not unlikely that the amount of the organic factor contained in the wheat and maize of Ration 3143 may have intensified the development of the rachitic changes by promoting the growth of the skeleton and increasing the duration of life of the animal, as did the small amount of butter fat in Ration 3133.

In a preliminary communication<sup>5</sup> Sherman and Pappenheimer described the production of rickets in rats by means of diets containing patent flour, 95 per cent; calcium lactate, 3 per cent; and sodium chloride, 2 per cent, with and without the addition of 0.1 per cent ferric citrate. They also described the prevention of the rickets when potassium phosphate in amounts equaling 0.4 per cent of the total was added. They also observed that, when no calcium was added to their faulty rations, osteoporosis instead of rickets developed. Patent flour is one of the most deficient foods which enters into the human diet, being exceeded in this respect only by isolated foods such as starch, sugars, fats, or polished rice. Bolted flour is rather poor in protein and this is of rather poor quality. It is very deficient in calcium, phosphorus, sodium, chlorine, iron, and possibly also in potassium. The only essential inorganic element which it probably contains in amount sufficient to meet the physiological needs of an animal is magne-

<sup>5</sup>Sherman, H. C., and Pappenheimer, A. M., *Proc. Soc. Exp. Biol. and Med.*, 1920-21, xviii, 193.

sium. Bolted flour is also very deficient in the antineuritic substance (water-soluble B) as shown by experiments on animals and by the frequent occurrence of beri-beri in man in Labrador and Newfoundland, where bread from this source is a principle article of food. It is exceedingly poor in fat-soluble A, and in the organic antirachitic factor. The lack of antiscorbutic substance in flour is, we believe, a matter of little or no importance in the nutrition of the rat, since this species is capable of synthesizing this complex. The ration employed by Sherman and Pappenheimer was, therefore, deficient in fat-soluble A, water-soluble B, its protein content, potassium, phosphate, and water-soluble C. In the presence of so considerable a number of defects in the ration it was impossible to be sure which were operative in the production of the disease. In the light of our experience it seems probable that their results should be interpreted as follows: The development of the rachitic condition in their animals was due (1) to a disproportion in the calcium-phosphate ratio, the calcium being present in optimal proportion or in a proportion not far from the optimal (3 per cent of the *lactate* of calcium) and the phosphorus at the lowest possible level, and (2) to a deficiency of the preventive organic substance. When neither calcium nor phosphate was added, the ratio between these (in the patent wheat flour) was more nearly the optimum than after the calcium addition, and, as was to be expected, osteoporosis, not rickets, developed. The animals needed (among other things) calcium very badly; yet we witness here the apparent paradox that meeting a physiological need and without exceeding it (*i.e.* by adding to the diet 3 per cent calcium lactate) more damage of a particular kind is done than would have resulted from permitting the pronounced calcium starvation in addition to the other deficiencies of their experimental diet to continue. *Apparently in the rat the profound disturbances in the deposition of lime salts in cartilage and bone and the changes in the cells of those tissues which give rise to the pathological complex known as rickets may be produced by disturbances in the diet of the optimal ratio between calcium and phosphorus in the absence of an amount of an organic substance contained in cod liver oil sufficient to prevent them. It would seem from the results of a large number of experiments, which will be published in detail soon, that in so far as calcium and phosphate are con-*

*cerned, the physiological relation in the diet between the two is of infinitely greater importance in insuring normal calcification than the absolute amount of the salts themselves.*

In examining the microscopic preparations from the rats which served as the material for the experiments reported in this paper, we were again struck with the similarity of behavior of the cartilage cell and of the bone corpuscle under the abnormal conditions imposed by the faulty diets. On coming into direct contact with the blood vessels the cartilage cell tended to undergo a gradual degeneration or transformation into a homogeneous substance indistinguishable except in its lack of a fibrillar arrangement from the osteoid zone surrounding the calcified trabeculae. On coming into less direct contact with the vascular elements the cartilage cell tended to revert to its original state in the undifferentiated cartilage, or to some intermediary state of development, or to undergo metaplasia into bone corpuscles, at least such as are found in the osteoid, or into connective tissue. In the process of reversion or of metaplasia of the cartilage cell in which it underwent a diminution in size, lost its characteristic staining reaction to hematoxylin, etc., it surrounded itself with (manufactured), or was surrounded by, an increased amount of homogeneous matrix, which remained free from lime salt deposition. The bone corpuscles in contact with the vascular elements, *i.e.* those of the osteoid borders of the trabeculae, underwent a diminution in size and a change in shape, and tended to revert, if they actually did not do so, to connective tissue cells. At the same time they surrounded themselves with (manufactured), or were surrounded by, an excessive amount of matrix, which remained free from lime salt deposition. In our experience whatever the reaction of the osteoblasts to the abnormal condition of the faulty diet, the same reaction will be manifested by the cartilage cell. The processes of bone resorption so prominent in the skeleton of rats confined for periods of some duration to faulty diets low in calcium seemed to be held in abeyance under the particular abnormal conditions obtaining in these experiments. Only slight evidences of resorptive activity in the calcified portions of the trabeculae could be found. In the presence of the particular disproportion in the calcium-phosphate ratio characterizing the faulty diets used in these experiments both osteoblast and cartilage cell seemed to undergo changes which made them particularly resistant to the destructive forces ordinarily in operation.

## EXPLANATION OF PLATES.

## PLATE 4.

FIGS. 1 and 2. Photomicrographs of low magnification of sections from the distal end of the femur of rats on the diet of Lots 3143 and 3133. The photomicrographs show the appearance of bones affected with a very severe rickets. There are broad zones of osteoid tissue around the trabeculae and the epiphyseal nucleus of ossification and the diaphysis. The cartilage is persistent to a marked degree, forming a wide metaphysis. It contains no calcium salts and is invaded by blood vessels from the shaft. (Rats 679 and 811. Diet of Lots 3143 and 3133.) Objective—Leitz microsummar F-5 full aperture. No ocular.

## PLATE 5.

FIG. 3. This picture shows a condition which exactly simulates that found in mild cases of rickets in man. The osteoid borders of the trabeculae in this bone are much narrower than those of the bones shown in Figs. 1 and 2. The cartilage is persistent and irregularly invaded. There is no zone of provisional calcification. The metaphysis of the bone is narrow and contains small scattered deposits of calcium salts. This picture was taken with the same apparatus used to photograph Figs. 1 and 2. (Rat 642. Diet of Lot 3127.)

FIG. 4. To show detail of the growing region of the bone in Fig. 3. Note the absence of provisional calcification and the irregular prolongation and invasion of the cartilage. The metaphysis, which is narrow, is made up of trabeculae of osteoid, and the metaphysis contains irregular deposits of lime salts. Objective—Leitz acromatic No. 3. No ocular.

## PLATE 6.

FIG. 5. Photomicrograph with the same apparatus used in making Fig. 4. This picture shows the metaphysis in detail of a bone affected with exaggerated rickets. The very broad metaphysis is composed of osteoid trabeculae, traversed by a few small marrow spaces, and blood vessels which have sprouted from the vascular tree of the diaphysis. Much of this osteoid tissue was the product of metaplasia of the cells of the epiphyseal cartilage, some of which may be seen embedded in it in a condition of transition into osteoid corpuscles. (Diet of Lot 3133.)

FIG. 6. High power photomicrograph. This picture shows calcified bone, *OS*, surrounded by a zone of osteoid tissue, *OST*. Note the lamination of the osteoid, the small size and wide separation of the osteoid corpuscles and the endothelioid appearance of the osteoblasts which surround the trabeculae, *O*. (Diet of Lot 3143.) Objective—Leitz acromatic No. 6. No ocular.

PLATE 7.

FIG. 7. This picture shows the cartilage cells in the metaphysis during their transformation into osteoid. In it the fusion of the cells into osteoid tissue in the immediate vicinity of an invading blood vessel is well shown. (Diet of Lot 3133.)

FIG. 8. Section from the distal end of the femur of a rat (No. 819) on the diet of Lot 3137. In this diet 2 per cent of cod liver oil replaced 1.5 per cent of dextrin and 0.5 per cent of butter fat. This bone was in a condition of osteoporosis with essentially normal calcification. There is a provisional calcified zone and no metaphysis has been formed. Only physiological osteoid tissue is present.

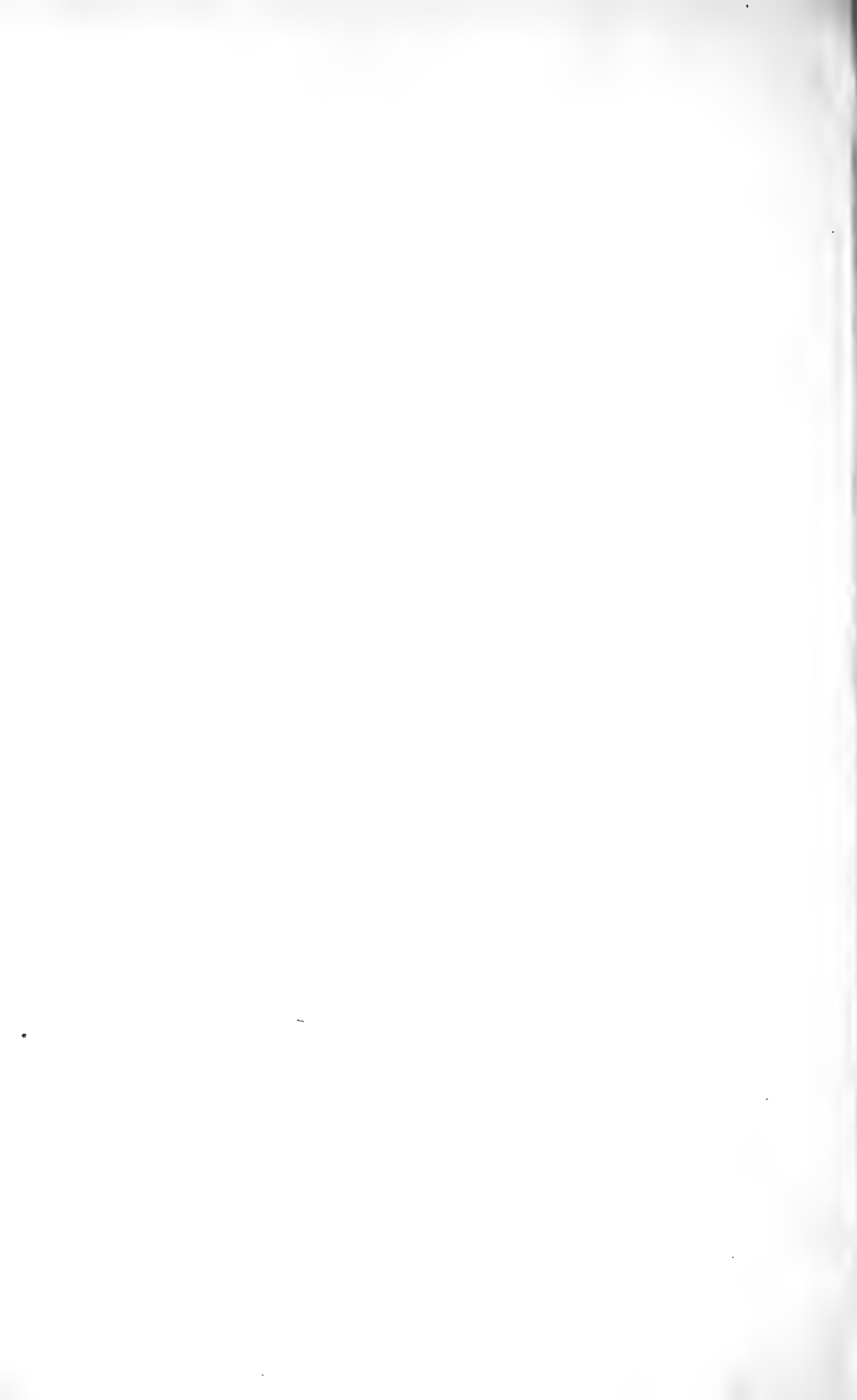




FIG. 1.

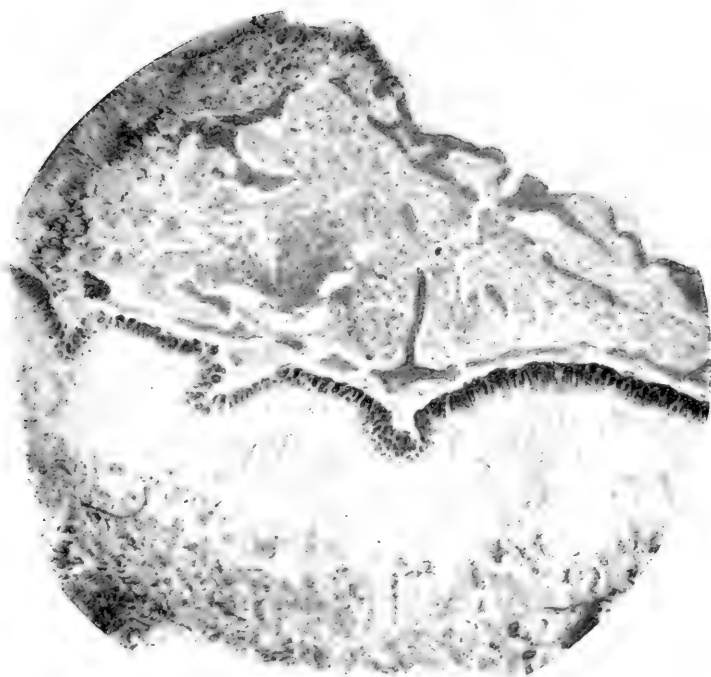


FIG. 2.







FIG. 3.



FIG. 4.

(McCollum, Simmonds, Shipley, and Park: Experimental rickets. VIII.)





FIG. 5.

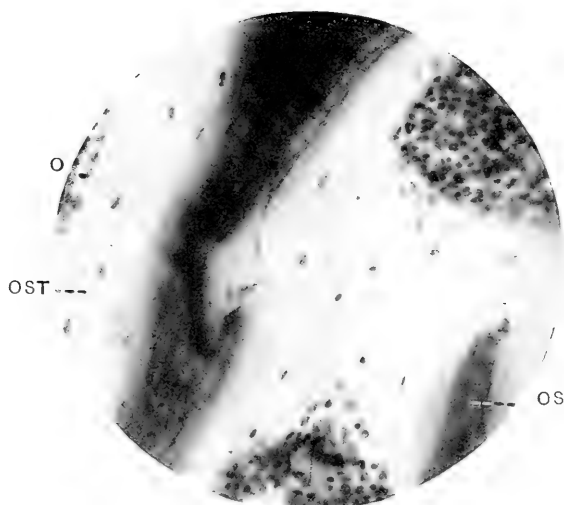


FIG. 6.

(McCollum, Simmonds, Shipley, and Park: Experimental rickets. VIII.)





FIG. 7.



FIG. 8.

(McCollum, Simmonds, Shipley, and Park: Experimental rickets. VIII.)



# THE DIFFUSIBLE CALCIUM OF THE BLOOD SERUM.

## I. A METHOD FOR ITS DETERMINATION.

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(Received for publication, June 22, 1921.)

It is now well recognized that the calcium in the plasma does not all exist in the form of simple solution. The first conclusive demonstration of a non-diffusible calcium we owe to Rona and Takahashi (1), who subjected serum to dialysis in which all inorganic salts except the calcium were fully compensated for in the dialyzing fluid outside the membrane, while calcium was present in varying amounts. By such means these authors showed that on the average only 65 per cent of the calcium in the serum was capable of diffusing through a membrane. Very interesting experiments on the state in which calcium occurs in serum were done by Cushny (2), who filtered ox serum through collodion membranes under 150 mm. mercury pressure. He found that, while sodium, potassium, and chlorine filtered as any ordinary solution would, a portion of the calcium was held back. The non-filterable (colloidal) calcium represented 30 to 40 per cent of the total.

This colloidal, non-diffusible calcium is looked upon as a protein combination and while nothing is known regarding its real nature, it seems definite enough to warrant further study. It seemed to us worth knowing whether in conditions of abnormal calcium metabolism, such as rickets or tetany, the amount of the diffusible and non-diffusible calcium might throw any light on the processes involved. Brinkman (3) and Brinkman and Van Dam (4) have recently published observations on the calcium ion concentration in serum and emphasize strongly the importance of

its exact maintenance. According to Brinkman, this maintenance of calcium ion concentration depends on the concentration of hydrogen ion and bicarbonate ion. It appeared to us that the amount of the total calcium in solution (diffusible, non-colloidal) might be influenced by differences in  $[H^+]$  or  $[HCO_3^-]$ , since it is perfectly well known that definitely acidifying the serum (as in Lyman's method of calcium determination) results in the total calcium being present in true solution.

In this paper we describe the technique of dialysis together with observations on variation in  $CO_2$  tension under which the dialysis took place, while in the following paper we record the results obtained on the blood in rickets and experimental tetany.

TABLE I.  
*Showing Dialysis Complete in 24 hours.*

Date and name.	Time of dialysis.	$CO_2$ saturation, $PCO_2$ mm. Hg.	Ca in serum in 4 cc.		Ca in dialysate in 4 cc.		Serum Ca per 100 cc.	Dialyzable Ca.	Remarks.
			Start.	End.	Start.	End.			
	hr.	mm.	mg.	mg.	mg.	mg.	mg.	per cent	
Jan. 20, 1921. A.M.P.	24	46.2	0.420	0.384	0.168	0.236	10.5	72	Duplicates.
	48	46.2	0.420	0.382	0.168	0.226	10.5	68	
	24	48.3	0.420	0.396	0.209	0.248	10.5	68	"
	48	48.3	0.420	0.394	0.209	0.246	10.5	67	
	24	46.0	0.420	0.414	0.258	0.256	10.5	60	"
	48	46.0	0.420	0.408	0.258	0.266	10.5	65	

In order to carry on our studies, we have devised a method of dialyzing serum against a buffer solution of the Ringer type, at the same time maintaining a constant  $CO_2$  tension in the dialyzing system.

If serum be dialyzed against a calcium-free Ringer buffer solution, there appears to be a progressive dissociation of the colloidal calcium compound, so that calcium continues to diffuse out, until at the end of 7 days 90 per cent has passed into the dialysate. When, however, calcium is added to the dialyzing fluid ("compensation dialysis"), equilibrium is obtained in 24 hours, as Table I shows.

Our method will now be described in detail.



# *Details of Method.*

*Collection of Blood for Dialysis.*—Blood is obtained by venous puncture into 100 cc. centrifuge tubes, defibrinated by whipping, centrifuged, and the serum pipetted. If not used immediately, the serum is kept stoppered in the ice box without preservative.

*Dialyzing Fluid.*—Fluid A, used in the early part of the work, was composed of equal volumes of double strength calcium-free Ringer's solution, and M/375 primary and secondary phosphates, having an initial pH of 7.1. The composition of the double strength Ringer's solution was:

NaCl.....	36.0	gm.
NaHCO <sub>3</sub> .....	10.08	"
KCl.....	1.68	"
Distilled H <sub>2</sub> O to.....	2,000	cc.

The composition of the phosphate solution was:

M/15 KH <sub>2</sub> PO <sub>4</sub> .....	15.2	cc.
M/15 Na <sub>2</sub> HPO <sub>4</sub> .....	64.8	"

This was diluted 25 times, so that the final concentration of phosphorus was approximately that of inorganic phosphate of blood serum, namely 0.003 gm. per 100 cc.

The hydrogen ion concentration of the Ringer phosphate solution after saturation with 6 per cent CO<sub>2</sub> was found to be pH 7.4. Depression of freezing point = 0.708 (18 per cent hypertonic).

Fluid B was based on analytical figures for normal human plasma obtained from Dr. Greenwald, to whom acknowledgment is hereby made for placing at our disposal his unpublished data, and its composition is given in Table II. Depression of freezing point of this fluid was 0.585 (isotonic). This solution was made up double strength, so that it could be mixed with equal volumes of double strength CaCl<sub>2</sub> solutions, as will be described under the method of compensation. The figures in the table represent the value of the single strength solution, ready for use. Calcium figures are not given, for these vary with the compensation desired. It will be noted that the values approximate closely those for normal human plasma, with the exception of the chlorine, which is here in excess of that found normally. The buffer action of this fluid is due mainly to its bicarbonate content. The curve of Fig. 1 illustrates the CO<sub>2</sub> dissociation of the fluid.

TABLE II.  
*Composition of Dialyzing Fluid.\**

Compound.	Cl	Na	K	Mg	P	Molecular concentration.
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
NaHCO <sub>3</sub> , 252 mg. ....		69.0				0.03
KH <sub>2</sub> PO <sub>4</sub> , 8.8 mg. ....			2.52		2.0	0.00065
MgCl <sub>2</sub> , 10.59 mg. ....	7.89			2.7		0.0011
KCl, 29.6 mg. ....	14.1		15.5			0.00397
NaCl, 625 mg. ....	380.0	246.0				0.1069
Totals. ....	401.99	315.0	18.02	2.7	2.0	

\*Based on analytical figures for human plasma obtained from Greenwald.

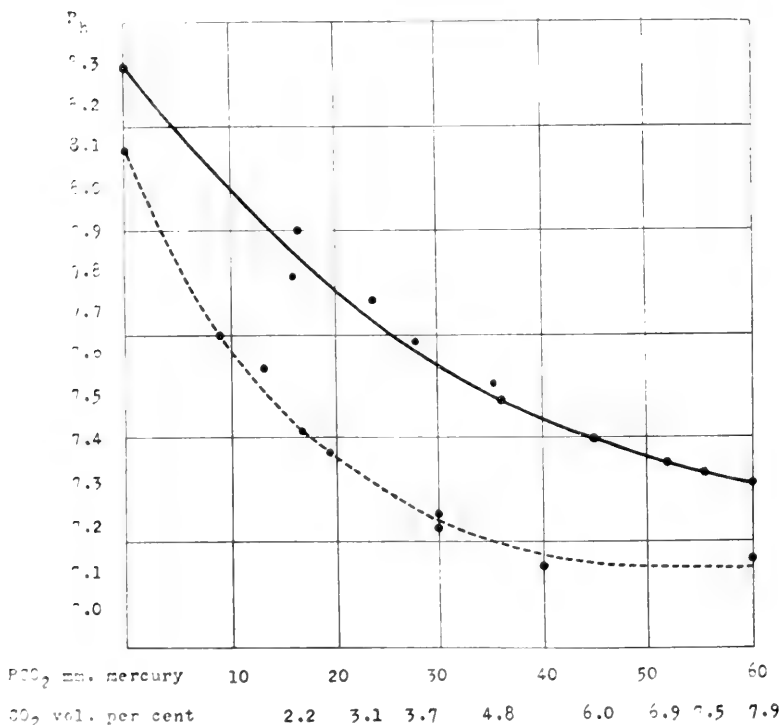


FIG. 1. CO<sub>2</sub> dissociation curve for dialyzing fluid B containing calcium. — shows curve of Fluid B. - - - represents the relation between pH and CO<sub>2</sub> tension in cat's blood, taken from Dale and Evans.

*Dialyzing Sacs.*—There was considerable difficulty in obtaining satisfactory sacs. Collodion membranes did not hold back protein for a sufficient length of time, and there was a progressive passage of fluid into the sac, presumably an osmotic phenomenon. Later, parchment sacs were obtained. These were Schleicher and Schüll's Diffusions-Hülsen, No. 579A. They proved to be impermeable to protein after 7 days dialysis at ice box temperature, and after 3 days at 48 to 52° C. A series of six sacs was tested for individual variations in permeability, using 0.9 per cent sodium chloride against distilled water. No significant differences in rate of diffusion of chlorine ion were found. Nor was there passage of fluid into the sac containing serum as determined by inserting a graduated capillary tube into the fluid within the sac. Before using, the sacs were soaked over night in 5 per cent hydrochloric acid and washed free of acid with distilled water, and allowed to dry in an open flask covered with filter paper. The same procedure was followed with the used sacs, so as to remove all possible traces of calcium in the sac wall.

*Pipettes.*—To obtain greater accuracy, special pipettes to conform to the requirements of the Bureau of Standards, were made for us by E. Leitz, Inc. These pipettes, measuring 4 cc. to the tip, and graduated in  $\frac{1}{16}$  cc. are straight with inside diameter of 5 mm. They were used for measuring the serum and dialyzing fluid before and after dialysis.

*Dialyzing Tubes.*—Special Pyrex glass tubes were made for this work,  $3\frac{1}{2} \times 1$  inches. The parchment sacs were suspended in these by means of cotton threads, threaded through the upper rim of the sac. The threads were looped around glass rings encircling the upper end of the sac, so as to prevent the sac from leaning against the tube, and so allowing the dialyzing fluid to creep up the sac wall.

*Regulation of CO<sub>2</sub> Tension.*—Before dialysis, both serum and dialyzing fluid were saturated with CO<sub>2</sub> of known tension, and this tension was maintained throughout the duration of the dialysis. CO<sub>2</sub>-air mixtures of any desired proportion were obtained by driving measured volumes of air and CO<sub>2</sub> through separate gas meters fitted with precision gauges, into a 20 liter rubber bag. After allowing time for diffusion, the CO<sub>2</sub> content of the mixture

was checked with a Fredericia apparatus. The composition as determined by analysis usually varied from the theoretical by less than 0.2 volume per cent. Where the error was greater than this, the bag was emptied and refilled. For analysis, a volume of approximately 1,500 cc. was passed through the apparatus.

Saturation of the dialysate and of the serum was performed by exposing 5 cc. of fluid in a 500 cc. separatory funnel through which 1,500 cc. of the gas mixture were passed (over moist glass beads). The fluid was shaken for 2 minutes. Care was taken in transferring the fluid to the dialyzing tubes to avoid unnecessary exposure to the air.

*Method of Compensation.*—A calcium chloride solution was made up roughly to contain the same calcium concentration as serum. The calcium was quantitatively determined by Lyman's method (5) and checked by the titration method. A quantity of the solution representing the desired amount of calcium was pipetted into the dialyzing tube and evaporated to dryness, first on the water bath and then in the oven, and cooled in the desiccator. The calcium-free dialyzing fluid was then added to the dried calcium chloride. This way of adding the calcium seemed of great advantage, in that any desired amount of the calcium chloride could be pipetted and dried. It was at once found, however, that a large error had crept in and that the analytical figures for the calcium in the dialyzing system were too high. The following experiment was then performed. 3 cc. of the calcium chloride solution (= 0.273 mg. of calcium) were pipetted into the Pyrex dialyzing tube and dried as described. The dry residue was then dissolved in 3 cc. of the dialyzing fluid which had been saturated with 6 per cent  $\text{CO}_2$ -air mixture and gave a pH of 7.4. The clear solution was then analyzed for calcium. Duplicates so treated and analyzed gave these results.

Tube A, from which 2.5 cc. of solution were taken for analysis, showed 0.248 mg. of calcium; the amount of calcium in the original calcium chloride solution was 0.228 mg.

Tube B. In 2.6 cc. of the redissolved calcium chloride, 0.260 mg. of calcium was found; while 2.6 cc. of the original solution contained 0.237 mg.

This experiment showed us the source of our error. Calcium, even in so small amount as 0.02 mg. (Tube A) and 0.023 mg.

(Tube B) was dissolved from the glass during the drying process. So small an amount would hardly seem to vitiate any results, but when working with only 2 cc. of serum, which contains about 0.2 mg. of calcium, it introduces an error of 10 per cent.

To avoid this error, the calcium was added by mixing with the dialyzing fluid B an equal volume of calcium chloride standard solution. These solutions were made up in four different concentrations:

Solution A, to contain 40 per cent of the serum calcium, using 10.5 mg. per 100 cc. as an average figure. Analysis, 2 cc., 0.168 mg.

Solution B, to contain 50 per cent of the serum calcium. Analysis, 2 cc., 0.210 mg.

Solution C, to contain 60 per cent of the serum calcium. Analysis, 2 cc., 0.258 mg.

Solution D, to contain 70 per cent of the serum calcium. Analysis, 2 cc., 0.297 mg.

When these calcium chloride solutions (double strength) were added in equal volume to the double strength dialyzing fluid B, there was a slow precipitation of the calcium carbonate, and so the solutions were kept separate until immediately before being used. Saturation with the CO<sub>2</sub>-air mixture prevented this precipitation.

*Technique of Dialysis.*—4 cc. of saturated dialyzing fluid are pipetted from the separatory funnel into the dialyzing tube, which has been previously filled with the same CO<sub>2</sub> mixture as that used for saturation. It is then corked. The pH of the dialysate is determined with the excess of dialyzing fluid in the funnel by the colorimetric method of Levy, Rowntree, and Marriott (6). 4 cc. of the saturated serum are pipetted into a *dry* sac which is then suspended by the attached threads in the dialyzing fluid, but with the serum level above that of the outer fluid level. This is done because when the sac has become thoroughly moistened, it expands and the level inside falls, while that outside rises. The tube is then closed with a paraffined cork, through which passes a glass tube. The air in the tube is replaced by the CO<sub>2</sub>-air mixture, an outlet being provided by a slit in the cork, and the tube sealed with paraffin. The duplicate tube having been set up in the same way, the two are placed in a tightly stoppered museum jar, which is also filled with air-gas mixture, and sealed with paraffin. Fig. 2 shows the apparatus set up.

*Calcium Determinations.* As much of the serum and dialysate as it is possible to recover are pipetted (usually 3.5 to 3.6 cc.), the pH of the dialysate determined as before dialysis, and the calcium in each is determined. The clear dialysate was treated in exactly the same manner as the dialyzed serum. In every case, where protein had leaked through, the material was discarded.

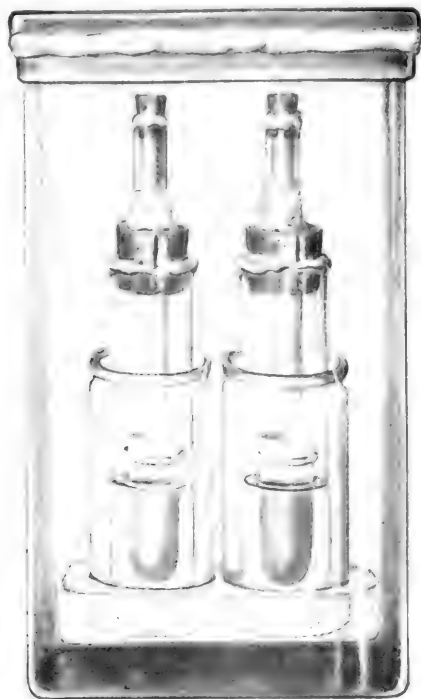


FIG. 2. Apparatus used in determination of diffusible serum calcium.

*Calculation of Results.* For this, the amount of calcium dialyzed, the calcium added to dialysate before dialysis, and the undialyzed serum calcium must be known. This is expressed in the formula

$$\text{Diffusible Ca} = \frac{(\text{Ca in dialysate after dialysis} \times 2) - \text{Ca added to dialysate}}{\text{Original serum Ca in amount used}}$$

Tables III to V show the results obtained. It will be seen in Table III that in a range of CO<sub>2</sub> saturation of serum from 17 mm. of mercury tension to 62 mm. there is no alteration in the percentage of diffusible calcium of the serum.

Table III also shows that a change in the hydrogen ion concentration of dialysate from pH 7.6 before dialysis to pH 7.0 after dialysis exerts no influence on the percentage of diffusible calcium.

TABLE III.  
*Varying CO<sub>2</sub> Tensions.*

Date and name.	CO <sub>2</sub> saturation, PCO <sub>2</sub> mm. Hg.	pH of dialysate.		Ca in serum in 4 cc.		Ca in dialysate in 4 cc.		Dialyzable Ca.	Total serum Ca per 100 cc.	Remarks.
		Before.	After.	Start.	End.	Start.	End.			
	mm.			mg.	mg.	mg.	mg.	per cent	mg.	
Feb. 2, 1921.	28.0	7.6	7.33	0.444	0.420	0.258	0.278	67	11.1	Dialyzing fluid was hypertonic.
A.M.P.	46.2	7.4	7.1	0.420	0.408	0.258	0.266	65	10.5	Fluid A.
	60.6	7.3	7.0	0.444	0.410	0.258	0.292	73	11.1	
Feb. 21, 1921.	17.6	7.6	7.5	0.440	0.448	0.295	0.296	67	11.0	Isotonic dialyzing fluid.
E.E.W.	43.5	7.45	7.35	0.440	0.424	0.295	0.306	72	11.0	
	62.0	7.2	7.0	0.440		0.295	0.300	70	11.0	Fluid B.

#### SUMMARY.

A method is described for determining diffusible serum calcium. Values are given for normal human and normal dog serum.

#### CONCLUSIONS.

1. The diffusible calcium of the serum of normal men and dogs was found to comprise from 60 to 70 per cent of the total serum calcium.

2. Varying the CO<sub>2</sub> saturation of the serum between 17 mm. mercury tension and 62 mm. does not alter this percentage.

TABLE IV.

*Dialyzable Calcium of Normal Bloods.*

Date and name.	CO <sub>2</sub> saturation.	pH of dialysate.		Ca in serum in 4 cc.		Ca in dialysate in 4 cc.		Dialyzable Ca.	Serum Ca per 100 cc.	Remarks.
		Be-fore.	After.	Start.	End.	Start.	End.			
1321	mm. Hg.			mg.	mg.	mg.	mg.	per cent	mg.	
Jan. 20, A.M.P.	46.2	7.4	7.1	0.420	0.408	0.258	0.266	65	10.5	Fluid A.
Feb. 8, E.C.K.	28.0	7.6	7.33	0.440	0.420	0.258	0.278	67	11.1	
Feb. 17, H.J.W.	46.3	7.4	7.3	0.421	0.398	0.258	0.272	67	10.5	Fluid B.
Feb. 21, E.E.W.	44.8	7.37	7.3	0.412	0.424	0.295	0.288	68	10.3	
Mar. 12, Mrs. S.	43.5	7.45	7.35	0.440	0.424	0.295	0.306	72	11.0	
Feb. 28, Dog 1.	45.5	7.43	7.25	0.416	0.420	0.295	0.300	73	10.4	
Mar. 3, Dog 2.	45.2	7.4	7.15	0.438	0.424	0.295	0.296	68	10.9	Considerable hemolysis.
Mar. 7, Dog 3.	43.7	7.4	7.0	0.444	0.434	0.295	0.302	69	11.1	Considerable hemolysis.
Mar. 21, Dog 4.	45.0	7.3	7.2	0.430	0.440	0.295	0.276	60	10.7	Slight hemolysis.
Mar. 24, Dog 5.	45.0	7.4	7.15	0.424	0.436	0.295	0.276	61	10.6	No hemolysis.
	43.7	7.4	7.15	0.408	0.384	0.254	0.268	69	10.2	“ “

TABLE V.

*Duplicate Analyses. Experimental Tetany, Dog 6.*

Series N.N.	Ca in serum in 4 cc.		Ca in dialysate in 4 cc.		Serum Ca in 100 cc.	Total Ca in system in 8 cc.			Dialyzable Ca. per cent
	Start.	End.	Start.	End.		Before.	After.	Difference.	
	mg.	mg.	mg.	mg.		mg.	mg.	mg.	
1	0.248	0.274	0.210	0.184	6.1	0.458	0.458	0.0	63
2	0.248	0.274	0.210	0.180	6.1	0.458	0.454	0.004	60
3	0.248	0.218	0.105	0.128	6.1	0.353	0.346	0.007	61
4	0.248	0.232	0.105	0.124	6.1	0.353	0.356	0.003	58



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## THE DIFFUSIBLE CALCIUM OF THE BLOOD SERUM.

### II. HUMAN RICKETS AND EXPERIMENTAL DOG TETANY.

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(Received for publication, June 22, 1921.)

In the preceding communication (1), there was reported in detail the technique employed in this work. It is our purpose in this paper to record the results of the study of the blood calcium in rickets and experimental tetany.

#### *Rickets.*

In the consideration of the pathology of rachitic bones and cartilage, there would seem to be four possibilities to account for the failure of proper ossification. First, that there is a deficiency of calcium in the diet or a failure of absorption of a sufficient supply; second, that there is an alteration of the form of the blood calcium, making it less available for the normal formation of bone; third, that there are absent intermediary products, possibly of the ductless glands, possibly other salts, particularly phosphates, which are necessary for the deposition of calcium in the bones; and fourth, that there is an alteration, chemical or otherwise, in the osteoid tissue and provisional cartilage cells or matrix, such that calcium, even though it may be supplied in sufficient and proper form by the blood, cannot be taken up by these cells. Or there may be a combination of any or all of these possibilities. It is to the second that we have directed particular attention.

Since the work of Howland and Marriott (2) in 1917, it has been known that there is a slight diminution of the serum calcium in rickets. They found in twenty-one cases an average of 9.4 mg. of Ca per 100 cc. of serum, whereas the normal is always

above 10 mg. Their lowest figure is 7.9 and in five other cases the Ca was less than 9 mg. We also have found a definite reduction of the serum calcium in rickets. Thus, five cases showed: 9.8, 8.7, 8.5, 9.0, and 7.6 mg. of Ca per 100 cc. of serum, respectively. The electrical reactions of these cases were normal, except in the first two, which showed anodal hyperexcitability.

The determination of the diffusible calcium in rachitic blood was carried out in precisely the same manner as that for normal blood described in our first paper.

Attention is directed to Table I, which gives the analytical figures. Both cases presented clinically, as well as by x-rays of the long bones, evidences of active rickets.

TABLE I.

CO <sub>2</sub> saturation P-CO <sub>2</sub> .	pH of dialysate.		Ca in 4 cc. of dialysate.		Ca in 4 cc. of serum.		Serum Ca per 100 cc.	Total Ca in system.			Dialyzable Ca.
	Be-fore.	After.	Be-fore.	After.	Be-fore.	After.		Be-fore.	After.	Difference.	
Baby L. F. Age 11 months.											
			mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	per cent
45.5	7.35		0.295	0.272	0.362	0.386	9.0	0.657	0.658	+0.001	68
45.5	7.4		0.295	0.268	0.362		9.0	0.657			66
45.5	7.4		0.210	0.210	0.362		9.0	0.572			58
Baby R. M. Age 12 months.											
44.6	7.4	7.4	0.295	0.244	0.302	0.334	7.6*	0.597	0.578	-0.019	63
45.0	7.35	7.3	0.295	0.246	0.302		7.6				65

\* Electrical reactions normal.

### *Tetany.*

It is hardly necessary to state that the blood calcium in infantile tetany and experimental tetany has been found markedly reduced. MacCallum and Voegtlin (3), MacCallum and Vogel (4), Howland and Marriott (2), Binger (5), and others are all agreed in this respect.

MacCallum, Lambert, and Vogel (6) in 1914 made the following statement: "If tetany blood be dialyzed under exactly the same conditions as normal blood, it still loses a proportionate amount of the calcium, which would perhaps show that it is not especially

the loss of diffusible calcium as contrasted with a non-diffusible form which is important in producing tetany."

We have sought in view of the increased  $\text{CO}_2$ -combining power of the plasma in tetany, reported by some investigators, to correlate the hypothesis discussed in the previous paper (1) with the low calcium content of tetany blood.

For this purpose, dogs were used, removing parathyroids, and in all but one case the thyroids as well.

The  $\text{CO}_2$ -combining power of the plasma in the tetany dogs was determined. Dog 4 had a preoperative combining power of 57.9 volumes per cent. During active tetany, it had fallen to 41.5 volumes per cent. Dog 6 showed practically no change. Dog 7 before operation showed 58 volumes per cent. 2 days later, without tetany, the combining power had risen to 69 volumes per cent. Later during a convulsion, it fell to 39 volumes per cent. In the presence of this definite acidosis, the diffusible calcium was still 66 per cent, so that it is apparent that Brinkman's hypothesis does not apply to the diffusible calcium.

*Dog 4.*—Female. Mar. 21. Blood was drawn under oil from jugular vein. The  $\text{CO}_2$ -combining power of the oxalated plasma, saturated with alveolar air, was 57.9 volumes per cent (Van Slyke micro method). Blood was also taken for determination of dialyzable calcium. This was not citrated, but was defibrinated by whipping (Table II).

Mar. 26. Under ether anesthesia, complete thyroparathyroidectomy was performed.

Mar. 28. Dog showed twitching of various groups of muscles, and the reflexes were hyperactive. Respirations were deep and rapid. Blood drawn at 10.30 a.m., before anesthesia, showed a plasma  $\text{CO}_2$ -combining power of 41.5 volumes per cent. Dog was then etherized, and blood drawn and defibrinated as before for determination of dialyzable calcium (Table II).

*Dog 6.*—Male. Apr. 4. Blood plasma  $\text{CO}_2$ -combining power was 57 volumes per cent, when saturated with 5.5 per cent  $\text{CO}_2$ -air mixture. The serum calcium was 10.2 mg. per 100 cc.

The dog was then etherized and complete thyroparathyroidectomy done.

Apr. 6. Early in the morning the dog was found in convulsions, and he had been vomiting. Respiration and pulse rates were very rapid. Slight twitchings of the abdominal muscles could be felt, but reflexes were not obtainable.  $\text{CO}_2$ -combining power was 54.1 volumes per cent when saturated with a 6.0 per cent  $\text{CO}_2$ -air mixture.

Blood was taken for determination of dialyzable calcium, Table III.

TABLE II.

*Dog 4.*

CO <sub>2</sub> saturation P-CO <sub>2</sub> .	pH of dialysate.		Ca in 4 cc. of dialysate.		Ca in 4 cc. of serum.		Serum Ca per 100 cc.	Total Ca in system.			Dialyzable Ca.
	Be-fore.	After.	Be-fore.	After.	Be-fore.	After.		Be-fore.	After.	Difference.	
Before operation.											
			mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	per cent
45	7.4	7.15	0.295	0.276	0.424	0.436	10.6	0.710	0.712	+0.002	61
45	7.4	7.15	0.295	0.274	0.424	Lost.	10.6	0.710			59
45	7.37	7.25	0.258	0.252	0.424	0.440	10.6	0.682	0.692	+0.010	58

2 days after operation. Thyroparathyroidectomy.

45	7.4	7.25	0.210	0.184	0.254	0.282	6.35	0.464	0.466	+0.002	62
45	7.42	7.2	0.210	0.180	0.254	0.282	6.35	0.464	0.462	-0.002	59
45	7.4	7.25	0.105	0.136	0.254	0.230	6.35	0.359	0.366	+0.007	65
45	7.4	7.2	0.105	0.114	0.254	0.244	6.35	0.359	0.358	-0.001	48*

\* This is an exceptional figure, possibly due to variation in the sac used.

TABLE III.

*Dog 6.*

2 days after operation. Thyroparathyroidectomy.

CO saturation P-CO <sub>2</sub> .	pH of dialysate.		Ca in 4 cc. of dialysate.		Ca in 4 cc. of serum.		Serum Ca per 100 cc.	Total Ca in system.			Dialyzable Ca.
	Be-fore.	After.	Be-fore.	After.	Be-fore.	After.		Be-fore.	After.	Difference.	
			mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	per cent
45.7	7.4	7.15	0.210	0.184	0.248	0.274	6.1	0.458	0.458	0.0	63
45.7	7.38	7.2	0.210	0.180	0.248	0.274	6.1	0.458	0.454	-0.004	60
45.7	7.4	7.2	0.105	0.128	0.248	0.218	6.1	0.353	0.346	-0.007	61
45.7	7.42	7.2	0.105	0.124	0.248	0.232	6.1	0.353	0.356	+0.003	58

TABLE IV.

*Dog 7.*

4 days after operation. Thyroparathyroidectomy.

CO <sub>2</sub> saturation P-CO <sub>2</sub> .	pH of dialysate.		Ca in 4 cc. of dialysate.		Ca in 4 cc. of serum.		Serum Ca per 100 cc.	Total Ca in system.			Dialyzable Ca.
	Be-fore.	After.	Be-fore.	After.	Be-fore.	After.		Be-fore.	After.	Difference.	
			mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	per cent
45	7.43	7.3	0.210	0.194	0.270	0.288	6.7	0.480	0.482	+0.002	66
45	7.42	7.25	0.210	0.196	0.270	0.286	6.7	0.480	0.482	+0.002	67
45	7.42	7.2	0.180	0.186	0.270	0.268	6.7	0.450	0.454	+0.004	71

*Dog. 7.*—Male. Apr. 9. CO<sub>2</sub>-combining power of plasma saturated with 6 per cent CO<sub>2</sub>-air mixture was 58 volumes per cent, and the serum calcium 10.3 mg. per 100 cc. Under ether anesthesia, the right thyroid, both upper parathyroids, and right lower parathyroid were removed.

Apr. 11. Dog showed slightly hyperactive knee jerks, and a CO<sub>2</sub>-combining power of 69.2 volumes per cent (saturated with 6 per cent mixture).

Apr. 12. Left thyroid and remaining parathyroid were removed.

Apr. 13. At 9 a.m. the dog had a general convulsion. CO<sub>2</sub>-combining power of plasma, saturated with a 6 per cent CO<sub>2</sub>-air mixture was 39 volumes per cent. Dog was etherized and blood was drawn from the jugular vein by cannula and defibrinated for calcium determination, Table IV.

*Dog. 8.*—Male. May 2. Plasma CO<sub>2</sub>-combining power was 45 volumes per cent, and the serum calcium was 10.7 mg. per 100 cc. The dog was etherized and right thyroid, both right parathyroids, and left lower parathyroid were removed. The parathyroids were calcified.

TABLE V.

*Dog 8.*

14 days after operation. Partial thyroparathyroidectomy.

CO <sub>2</sub> saturation P-CO <sub>2</sub> .	pH of dialysate.		Ca in 4 cc. of dialysate.		Ca in 4 cc. of serum.		Serum Ca per 100 cc.	Total Ca in system.			Dialyz- able Ca.
	Be- fore.	Af- ter.	Be- fore.	After.	Be- fore.	After.		Be- fore.	After.	Differ- ence.	
			mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	per cent
44.7	7.4	7.25	0.210	0.214	0.336	0.336	8.4	0.546	0.550	+0.004	65
44.7	7.4	7.3	0.210	0.218	0.336	0.346	8.4	0.546	0.564	+0.018	67
45.5	7.42	7.3	0.127	0.182	0.336		8.4				71
45.5	7.4	7.35	0.127	0.182	0.336		8.4				71

May 10. The dog showed slight twitching of abdominal muscles and spasticity of legs.

May 11. Serum calcium was 7.2 mg. per 100 cc.

May 12. Condition noted on May 10 has persisted, but there have been no convulsions, nor active tetany.

The dog was etherized and blood taken for calcium determination as in previous experiments, Table V.

## SUMMARY.

In two cases of rickets, with serum calcium of 9.0 and 7.6 mg. per 100 cc., the percentage of diffusible calcium was found to be between 58 and 70 per cent, within the range found in normal subjects.

In four cases of experimental tetany in dogs, similar percentages of dialyzable calcium were found, 58 to 71 per cent, with serum calciums of 6.1 to 8.4 mg. per 100 cc. Two of these dogs showed a reduced  $\text{CO}_2$ -combining power of the plasma at the time the calcium determinations were made, showing that this form of acidosis does not affect the diffusible calcium. It, therefore, appears that, in so far as can be determined by *in vitro* experiment, the reduced serum calcium in experimental tetany is not due to a lowering of the diffusible as contrasted with the non-diffusible form. The proportion between the two remains constant in the presence of a reduced total. Also, this proportion does not change with varying  $\text{CO}_2$ -combining powers of the plasma.

#### CONCLUSIONS.

The diffusible calcium of the serum in experimental tetany in the dog and human rickets is between 60 and 70 per cent of the total serum calcium.

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## THE OXYGEN DISSOCIATION OF HEMOGLOBIN, AND THE EFFECT OF ELECTROLYTES UPON IT.

BY EDWARD F. ADOLPH AND RONALD M. FERRY.

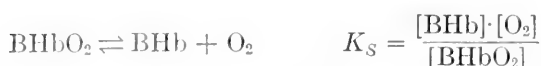
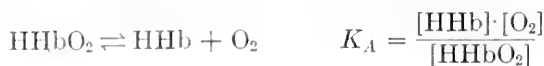
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(Received for publication, June 21, 1921.)

Barcroft (1) has gathered extensive data on the dissociation of oxyhemoglobin. Plotting oxygen tension against percentage saturation with oxygen, he has drawn dissociation curves, and, by developing the aggregation theory in conjunction with Hill (2), he has sought to explain divergences in their form. Incidentally they have represented mathematically the curves for the dissociation  $\text{HbO}_2 \rightleftharpoons \text{Hb} + \text{O}_2$  by a general equation  $K = \frac{[\text{Hb}] \cdot [\text{O}_2]^n}{[\text{HbO}_2]}$  and have determined constants which fit the equation in the case of every experimental curve.

The data of Barcroft and Camis (3) have shown that in whole blood divergences in form of the curves are due to variations in the salt content of the blood from various species and individuals; but that, on the other hand, preparations of crystallized hemoglobin made at different times give sensibly similar curves. Barcroft and Roberts (4) have demonstrated that dialyzed hemoglobin gives curves which differ from those for crystallized hemoglobin, but which are identical for dialyzed preparations from different animals. These curves they have regarded as representative of pure hemoglobin. Barcroft and Means (5) have shown that carbonic acid shifts the dissociation curve of dialyzed hemoglobin and have measured the shift quantitatively.

Barcroft (1) and L. J. Henderson (6) have suggested that hemoglobin in alkaline solution, or alkali hemoglobinate, has a smaller dissociation constant for oxygen than has acid hemoglobin. And Henderson has by an indirect method calculated the ratio of two constants for the following equations in the case of whole blood:



showing that  $K_A$  is greater than  $K_S$ . Henderson concludes: ". . . oxyhemoglobin must be a stronger acid than reduced hemoglobin. Of course it also follows that the salts of hemoglobin must have a greater affinity for oxygen than has acid hemoglobin itself."

To obtain direct experimental evidence upon this point, the effect of electrolytes and non-electrolytes on the dissociation curve of oxyhemoglobin was studied by us at the suggestion of Professor L. J. Henderson.

### *Methods.*

Fresh defibrinated beef blood was centrifuged, and the corpuscles thrice washed with 0.9 per cent sodium chloride solution. The corpuscular magma, placed in freshly made collodion sacs of about 100 cc. capacity, was dialyzed against running tap water for 48 hours. Dialysis was continued on ice for one or more days against several changes of distilled water. The sacs were usually closed securely to diminish dilution of the contents.

In order to rid the hemoglobin of metallic cations one of the following procedures, suggested by Professor Henderson, was usually adopted. Either (*a*) the solution was poured from the sacs and saturated with carbon dioxide, dialysis being continued against distilled water; or (*b*) the sacs were placed intermittently in distilled water saturated with carbon dioxide. In both cases the hemoglobin was finally dialyzed against pure distilled water. It was supposed that this treatment with carbonic acid would free hemoglobin from the cations at its isoelectric point, according to the equation  $\text{BHb} + \text{H}_2\text{CO}_3 \rightleftharpoons \text{BHCO}_3 + \text{HHb}$ .

The freedom of the dialyzed solutions from electrolytes was roughly estimated by measuring conductivity at 25°C. The lowest conductivity reached,  $8 \times 10^{-5}$  reciprocal ohms, is the lowest conductivity reported in the literature when it is considered that the concentration of the hemoglobin was 16 per cent. Rarely did the conductivity exceed  $3 \times 10^{-4}$  reciprocal ohms,

which is 4 per cent of that of whole blood, or equivalent to that of a 0.0027 M potassium chloride solution.

The hemoglobin solutions contained, on the average, 14 percent hemoglobin, as measured by the oxygen combined with the hemoglobin after saturating it with pure oxygen at atmospheric pressure, and assuming a molecular weight of 16,700. Except for a diminution in conductivity, no difference could be detected in the behavior of solutions which had been treated with carbon dioxide during dialysis as compared with those not treated.

The solutions of hemoglobin were equilibrated with mixtures of carbon dioxide-free hydrogen and of air, selected to give about 50 per cent saturation of the hemoglobin with oxygen. This was done in a rubber-stoppered bottle of 1 liter capacity, provided with two outlet tubes. Through the shorter, a sample of solution could be withdrawn without exposure to the outside air; the other was provided for gas sampling. 20 to 25 cc. of hemoglobin solution, evacuated by boiling with a water pump at 35 to 40°C., were run in from a pipette to the bottle, which had been previously filled with the gas mixture. Electrolytes and non-electrolytes, in solutions of suitable concentration were introduced through the rubber stopper from a graduated Luer syringe. The equilibrator bottle was then rotated continuously for 15 to 30 minutes in a thermostat at 38°C. The rotation was interrupted to adjust the pressure to that of the atmosphere. The same solution was often equilibrated five times.

After equilibration the bottle was inverted in order to collect the solution in its neck, thereby presenting only a small surface to the gas above it. A gas sample of 10 cc. was then taken into an analyzer of the type described by Y. Henderson (7), and analyzed for carbon dioxide and oxygen. Some of the stock gas mixture was then admitted into the bottle at atmospheric pressure and room temperature, and a 2 cc. sample of solution run into a Mohr pipette. The oxygen content was determined by the blood-gas method of Van Slyke (8). A second sample of solution was saturated with pure oxygen at atmospheric pressure and room temperature in a separatory funnel, and the oxygen capacity was determined. This was determined once or twice in a series of successive equilibrations. Van Slyke's method is not well suited to measuring the percentage saturation of hemoglobin with oxygen,

since two samples must be taken for each determination. The results of many workers seem to show that the quantities of oxygen found, though consistent among themselves, are too high. It was, however, the most available method. Great care was taken to correct calculations upon each analysis for dissolved gases, and to determine the errors due to manipulation and reagents.

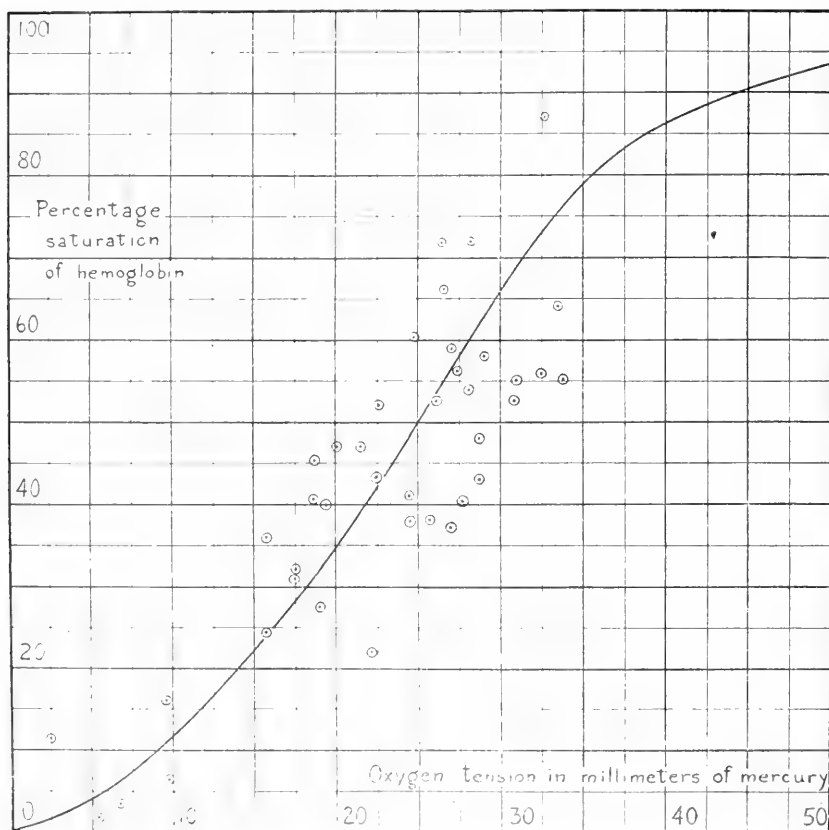


FIG. 1.

## EXPERIMENTAL RESULTS.

*Dialyzed Hemoglobin.*—Some 40 determinations of percentage saturations were made upon 26 different solutions prepared from the blood of 4 animals. These are plotted against oxygen tension

in Fig. 1, and an arbitrary curve has been drawn through these points. It is a reflexed or S-shaped curve, and resembles Bohr's (9) dissociation curve for crystallized hemoglobin.

Blood samples from the same animal prepared simultaneously gave solutions exhibiting the most uniform results. There is, however, no close agreement among the other points, nor was any to be expected from the varying conditions. Most of the points to the right of the line were obtained in the latter part of the investigation, and it is probable that these points are to be explained by the fact that the gas analyses occasionally revealed small tensions of carbon dioxide, 1 to 8 mm., which were reckoned in with the oxygen.

The individual points bear no demonstrable relation to the purity of the hemoglobin as measured by conductivity, nor to the use of carbon dioxide during dialysis. The variations among corresponding points may be due in part to differences in hydrogen ion concentration, to very slight differences in the electrolyte content, and to bacterial action. None of these factors was adequately controlled.

*Addition of Acid.*—Lactic acid and carbonic acid (Fig. 2, B and A) lowered the amount of oxygen combined at a given oxygen tension (30 mm.), and the effect was increased by increasing concentrations of acid. This change was reversible, and could be nearly restored by the addition of an equivalent amount of alkali. Concentration of lactic acid greater than 0.015 M caused a gradual diminution of oxygen capacity due to an irreversible change in the hemoglobin.

Barcroft and Means (5) have demonstrated this effect of carbonic acid in varying concentrations upon dialyzed hemoglobin. Barcroft and Orbeli (10) have shown that lactic acid lowers the amount of oxygen taken up by whole blood.

*Addition of Alkali.*—Solutions of sodium hydroxide (Fig. 2, B) from 0.001 to 0.04 M caused more oxygen to be taken up by the hemoglobin at a given tension. This change was reversible upon the addition of an equivalent amount of acid. It increased with the amount of alkali added. Similar results were obtained with disodium phosphate,  $\text{Na}_2\text{HPO}_4$ , from 0.01 to 0.1 M (Fig. 2, D). These results confirm those of Barcroft and Camis (3) with ammonia, disodium phosphate, and sodium bicarbonate added

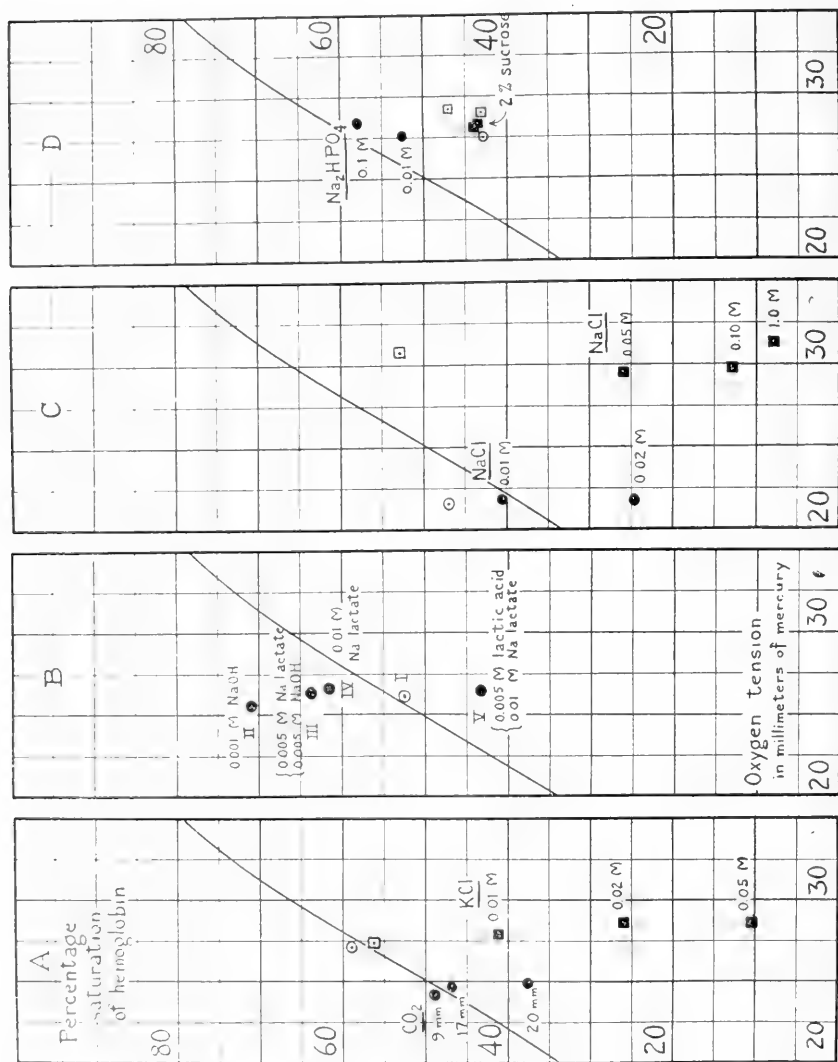


Fig. 2.

to crystallized hemoglobin; and those of Momose (11) with sodium hydroxide and ammonia added to whole blood.

*Addition of Neutral Salts.*—Potassium and sodium chlorides at concentrations from 0.01 to 1.0 M reduced the oxygen bound at a given oxygen tension (Fig. 2, A and C). This effect increased with increasing concentration, at first rapidly and then more gradually. A solution containing both sodium and potassium chlorides had the same effect as a solution of either alone.

*Addition of Non-Electrolytes.*—Neither urea from 0.1 to 1.0 M nor sucrose of 2 per cent (Fig. 2, D) affected the oxygen dissociation of the dialyzed hemoglobin solutions. These results confirm those for urea of Poulton and Ryffel (12) and of Momose (11) both of whom used whole blood.

#### SUMMARY.

Our results apparently differ from those of Barcroft in two respects. Our dialyzed solutions of hemoglobin give S-shaped curves comparable to those of Bohr (9) for crystallized hemoglobin. Barcroft's hyperbolic curves for dialyzed hemoglobin were obtained when ammonium carbonate or hydroxide was added,<sup>1</sup> and we have obtained comparable points when other alkalies were added. Barcroft has recently<sup>1</sup> obtained curves similar to ours for dialyzed hemoglobin to which no alkali was added.

Secondly, Barcroft (3) found that the addition of neutral salts to crystallized hemoglobin increased the amount of oxygen taken up by it at a given tension of oxygen. We have found the reverse to be the case with our samples of dialyzed hemoglobin. Very recently Barcroft<sup>1</sup> has made the same observation. Our results were obtained only at oxygen tensions of about 30 mm., and we are unable to predict the result at higher tensions.

#### DISCUSSION.

The chemical behavior of hemoglobin has been the subject of much speculation. The data of Barcroft (1); Douglas, Haldane, and Haldane (13); Roaf (14); and others, have been clearly interpreted on the basis of Hill's (2) aggregation theory as applied to

<sup>1</sup>We are greatly indebted to Mr. Barcroft for communicating the results of his recent experiments to one of us.

the mass action principle. The implications of the aggregation theory, which we believe serves as a foundation for understanding the chemistry of hemoglobin, have been ably set forth (1, 2, 13).

Up to the present time little has been said of the dependence of the equilibrium between oxygen and hemoglobin upon that between hemoglobin and electrolytes as such. The present experiments suggest that the degree of electrolytic dissociation of hemoglobin, as of other proteins (15), is variable, and that it is markedly different for alkali hemoglobins and for acid hemoglobin. The effect of acid in diminishing the amount of oxygen or carbon monoxide bound by hemoglobin at a given tension, the effect of alkali in increasing the saturation with oxygen at a given tension, and the fact that oxyhemoglobin behaves as if it were a more highly dissociated acid than reduced hemoglobin (6, 16, 17) give support to this view.

Certain facts are explicable upon both hypotheses, such as: the influence of neutral salts, the absence of effects due to non-electrolytes, and the increased osmotic pressure of hemoglobin in alkaline solution.

It seems probable that the electrolytic dissociation of hemoglobin must be considered as supplementary to its aggregation in giving to hemoglobin its remarkable range of variation in physico-chemical activity. The conception of hemoglobin as an ionized substance is the only view so far suggested to account for the effects of acid and of alkali upon hemoglobin.

Unfortunately the present data are inadequate for more than a qualitative discussion. It is hoped that work now in progress will give a quantitative definition of the effects due to ionic equilibria.

We are indebted to Professor L. J. Henderson for much valuable aid and criticism.

#### CONCLUSIONS.

1. A technique is described for dialyzing hemoglobin, depending upon its protein property of acting as a dissociable electrolyte.
2. Hemoglobin prepared by several procedures for dialysis gives S-shaped curves for oxygen dissociation.
3. Addition of alkali with formation of alkali hemoglobinate results in an increase of the oxygen bound by hemoglobin at a constant tension.



4. Neutral salts even in small concentrations decrease the amount of oxygen bound.
5. Non-electrolytes have no effect upon the oxygen equilibrium.
6. The equilibrium between oxygen and hemoglobin is a function of that between hemoglobin and electrolytes.

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# ANIMAL CALORIMETRY.

SEVENTEENTH PAPER.

## THE INFLUENCE OF COLLOIDAL IRON ON THE BASAL METABOLISM.

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(Received for publication, June 1, 1921.)

### INTRODUCTION.

The biological action of inorganic hydrosols has been extensively studied during the years which have elapsed since Credé<sup>1</sup> introduced the use of colloidal silver into medicine. From these investigations it seems very probable that the action of the inorganic hydrosols is much the same as the action of small doses of salts of the same metals (see the literature by Bechhold<sup>2</sup>).

The colloidal metal dissociates slowly in water into metal ions, and the bactericidal action, the property which has been the most studied, is supposed to be due to these dissociated ions. After intravenous injection the colloids are distributed all over the body within  $\frac{1}{2}$  to 1 hour and, while still in the colloidal state, are deposited in almost every organ with subsequent slow dissociation of ions.

Investigations on the influence of these substances upon the metabolism are very rare. It has been found that silver hydrosol increases the protein metabolism.<sup>3</sup>

A special interest is connected with the negative iron oxide hydrosol. While the positive iron oxide hydrosol, according to

<sup>1</sup> Credé, B., *Arch. klin. Chir.*, 1897, lv, 861.

<sup>2</sup> Bechhold, H., *Die Kolloide in Biologie und Medizin*, Dresden and Leipsic, 2nd edition, 1919.

<sup>3</sup> Bechhold,<sup>2</sup> p. 402.

Bechhold, unites with the negative blood colloids, forming an irreversible gel, the negative iron oxide hydrosol easily mixes with serum without producing any coagulation. This colloid solution is said to act as an oxygen carrier and to have properties similar to hemoglobin.<sup>4</sup>

The purpose of this investigation has been to examine the influence of such an intravenously injected colloidal iron solution on the basal metabolism.

#### *Methods.*

Two dogs, trained for calorimeter work, were used in the experiments. The dogs were fed once daily with the "standard diet."<sup>5</sup>

The bladder was emptied and washed before and after each experiment.

The hydrosol was injected into the jugular vein, and immediately thereafter the dog was placed in the calorimeter. In each experiment 5 cc.<sup>6</sup> were used.

The movements of the dog in the calorimeter were recorded on a smoked drum.

The efficiency of the calorimeter was controlled by frequent alcohol checks.

#### *Basal Metabolism.*

The standard diet was administered at 5 p. m. the day before the experiment. Thus, the basal metabolism was determined 18 to 20 hours after feeding.

In all experiments the dog was perfectly quiet, and the temperature of the calorimeter was between 25 and 26°C.

The basal metabolism of Dog 20 was found to average 14.92 calories per hour and the respiratory quotient 0.80 (Table I).

The basal metabolism of Dog 18 was found to be 16.18 calories per hour and the respiratory quotient 0.80.

#### *The Influence of Colloidal Iron Intravenously.*

The results of intravenous injection of 5 cc. of colloidal iron are given in Table II. The average total heat production per hour

<sup>4</sup> Bechhold,<sup>2</sup> p. 417.

<sup>5</sup> Lusk, G., *J. Biol. Chem.*, 1912-13, xiii, 185.

<sup>6</sup> Made by Laboratoires Clin. Paris.

of Dog 20 was 16.00 calories and the average respiratory quotient 0.84. One of the experiments, No. 10-A, was performed immediately after the basal metabolism was determined, on the same day.

After administering colloidal iron the heat production of Dog 18 was found to be 18.72 calories per hour and the respiratory quotient 0.82.

TABLE I.  
*Basal Metabolism of Dog 20.*

Date.	Experiment No.	Indirect calorimetry.	R. Q.
<i>1921</i>			
Apr. 7.....	3	14.45	0.81
May 6.....	5	14.77	0.79
" 9.....	7	14.94	0.81
" 13.....	10	15.25	0.78
" 14.....	11	15.22	0.81
Average .....		14.92	0.80

TABLE II.  
*Colloidal Iron Intravenously—Dog 20.*

Date.	Experiment No.	Indirect calorimetry.	R. Q.
<i>1921</i>			
Apr. 8.....	4	15.63	0.85
May 7.....	6	15.79	0.87
" 13.....	10-A	16.40	0.81
Average .....		16.00	0.84

The total heat production was thus increased about 7 per cent in Dog 20 and about 15 per cent in Dog 18.

The average CO<sub>2</sub> output per hour of Dog 20 after the injection was 5.51 gm., but only 4.89 gm. in the basal metabolism. The average O<sub>2</sub> consumption was 4.68 gm. per hour after injection and 4.46 gm. in the basal metabolism.

In Dog 18 the CO<sub>2</sub> production was increased from 5.37 to 6.35 gm. per hour and the O<sub>2</sub> consumption from 4.87 to 5.63 gm. per hour after the injection.

TABLE III.

Dog 20.

Standard diet given at 5 p.m. the previous day. The results are expressed in average values per hour.

Date.	Experiment No.	Time.	CO <sub>2</sub>	O <sub>2</sub>	R. Q.	H <sub>2</sub> O	Urine N.	Calories.			Remarks.
								Protein.	Non-protein.	Total heat calculated.	
1921 Apr. 7	3 Basal.	11.22-2.22	gm. 4.80	gm. 4.33	0.81	gm. 11.06	gm. 0.082	2.18	12.27	14.45	Alcohol check Apr. 4, average R. Q. 0.665. Weight 8.47 kg. Dog quiet.
"	4 Colloidal iron intravenously.	11.32-2.32	5.52	4.65	0.85	12.86	0.095	2.52	13.11	15.63	Weight 8.47 kg. Colloidal iron intravenously, 5 cc. Dog quiet.
May 6	5 Basal.	11.18-2.18	4.77	4.40	0.79	12.15	0.098	2.62	12.15	14.77	Alcohol check May 5, average R. Q. 0.667. Weight 9.20 kg. Dog quiet.
"	6 Colloidal iron intravenously.	11.57-1.57	5.58	4.47	0.87	11.47	0.105	2.80	12.99	15.79	Weight 9.10 kg. Colloidal iron intravenously, 5 cc. Dog quiet.
"	7 Basal.	12.02-2.02	4.92	4.39	0.81	11.12	0.098	2.60	12.34	14.94	Weight 9.10 kg. Dog quiet.

May 13	10 Basal.	11.10- 1.10	4.91	4.60	0.78	15.43	0.071	1.90	13.35	15.25	Alcohol check May 10, average R. Q. 0.668. Weight 9.30 kg. Dog quiet.
" 13	10-A Colloidal iron intravenously.	2.40- 5.40	5.43	4.92	0.81	12.96	0.077	2.03	14.38	16.40	5 cc. colloidal iron intravenously 20 min. after the last experiment. Between these experiments the dog was catheterized, but received no food or drink. Dog quiet.
" 14	11 Basal.	10.25-12.25	5.04	4.57	0.81	11.32	0.079	2.09	13.13	15.22	Weight 9.20 kg. Dog quiet.

TABLE IV.  
*Dog 18.*  
 Standard diet given at 5 p.m. the previous day. The results are expressed in average values per hour.

Date.	Experiment No.	Time.	CO <sub>2</sub>	O <sub>2</sub>	R. Q.	H <sub>2</sub> O	Urine N.	Calories.			Remarks.
								Protein.	Non-protein.	Total heat calculated.	
1921 May 17	85 Basal.	12.25-2.25	gm. 5.37	gm. 4.87	0.80	gm. 7.86	gm. 0.107	2.85	13.33	16.18	Weight 9.45 kg. Dog quiet.
" 18	86 Colloidal iron intravenously.	11.36-1.36	6.35	5.63	0.82	12.37	0.137	3.65	15.07	18.72	Colloidal iron intravenously, 5 cc. Weight 9.45 kg. Dog quiet.



The respiratory quotient was slightly higher in both dogs during the injection experiments than during the determinations of the basal metabolism.

There was no marked increase of the protein metabolism. The chief increase of the total heat production fell on the non-protein metabolism.

The results are summarized in Tables III and IV.

#### CONCLUSIONS.

1. Intravenous injections of iron hydrosol in dogs cause an increase of the  $O_2$  consumption and the  $CO_2$  production. The average increase of the heat production in one of these dogs was 7 per cent and in one experiment on the other the increase was 15 per cent.

2. The increased metabolism coincides with a slight increase of the respiratory quotient.

3. The chief increase of the total heat production falls on the non-protein metabolism.

I wish to express my sincerest thanks to Professor Graham Lusk for advice and help in running the calorimeter, and to Mr. James Evenden for technical assistance.



## CHEMICAL FACTORS IN FATIGUE.

### I. THE EFFECT OF MUSCULAR EXERCISE UPON CERTAIN COMMON BLOOD CONSTITUENTS.\*

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(Received for publication, June 2, 1921.)

The beginning of the truly chemical study of muscular exercise and fatigue may be traced back to Du Bois Raymond who, in 1859, showed that contracting muscles become acid as a result of their activity. Others, following closely, pointed out that not only is lactic acid formed in the working muscle but there is a simultaneous disappearance of carbohydrates, notably glycogen, the partial oxidation of which presumably gives rise to the acid.

Space does not permit of an extended review of the work which followed from then till the present day, but a few of the outstanding features may be noted.

Ranke, in 1865, first investigated the effect upon the contractility of surviving, excised muscles produced by perfusion with solutions of various substances. His experiments were later extended and elaborated by Lee, Burridge, and others with the result that a number of substances were found whose influence upon surviving muscle produced to a certain extent the recognized phenomena of fatigue, as evidenced by a decrease in power of contraction. There have been reported from time to time among these so called "fatigue products" (some or all of which may possibly be formed in muscles during their activity and produce the condition of fatigue) the following: lactic and oxybutyric acids and their alkali salts,  $\text{HCl}$ ,  $\text{H}_2\text{SO}_4$ ,  $\text{CO}_2$ ,  $\text{NaH}_2\text{PO}_4$ ,  $\text{KH}_2\text{PO}_4$ , phenol, indole, skatole, and potassium ions.

\*A thesis presented to the Department of Chemistry of Leland Stanford Junior University in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

Weichardt, in 1904, claimed to have isolated a specific fatigue toxin, similar to the bacterial toxins, which produced all the symptoms of fatigue when injected into the blood stream. He purported furthermore to have isolated an antitoxin, by regular immunological methods, but his results have never been confirmed.

The excretion of substances in the urine during and following muscular work has received considerable attention.

It seems to be generally conceded that no increase in nitrogen metabolism accompanies muscular work. Nevertheless, a number of workers have reported substantial increases in nitrogen elimination for some time following a period of strenuous activity. The current opinion of the independence of nitrogen metabolism is therefore not without question. The commonly reported increases in sulfur and phosphorus elimination may also have a bearing on this point.

The question of changes in the excretion of nitrogenous extractions is still unsettled. Creatine and creatinine are apparently not involved in any regular way, but uric acid and the purines have in general been found to increase after work.

The acidity of the urine is generally, but not invariably, found to increase, as might be expected from the formation of lactic acid in the muscles and the lowering of the alkaline reserve of the blood.

The output of carbon dioxide is invariably increased in exercise, which is important when the demonstrated fatigue-producing action of carbon dioxide on the muscles is borne in mind.

Sugar is practically the only constituent of the blood which has been quantitatively followed during muscular exercise, but there is no agreement between the results from different sources.

A number of secondary physiological concomitants of fatigue have been reported, such as an increased number of red and of white corpuscles.

In summarizing the whole question we may lay down certain general principles: (1) Substances giving rise to hydrogen ions, such as lactic and oxybutyric acids, monopotassium phosphate, and carbon dioxide, are causal agents in fatigue. (2) Certain products of protein disintegration, indole, skatole, and phenol, are apparently capable of producing fatigue symptoms and may

be closely related to normal fatigue. (3) There is some evidence that certain negative ions, as the lactate and oxybutyrate, as well as certain positive ions, especially potassium, may be causal agents in fatigue. (4) There is no evidence substantiating Weichardt's theory, still largely quoted, of specific fatigue toxins. (5) The formation and excretion of a number of materials such as uric acid, purines, urea, etc., may be influenced by muscular work and fatigue, without their standing in any causal relation to fatigue.

#### EXPERIMENTAL.

The present study was undertaken with the purpose of determining, by means of improved biochemical methods of reasonable accuracy, the changes which occur in the more common constituents of the blood during fairly severe muscular exercise. The investigation will later be extended to other substances, and other conditions.

A certain amount of preliminary work was done with dogs as subjects, but the results were so irregular and the conditions of the experiments so difficult to control that it was entirely abandoned in favor of human subjects, nearly all of whom were university students. A few sets of results were obtained with running as a mode of exercise. These data, as far as they go, are given in Table I. Later it was deemed advisable to lengthen the period of exercise and two subjects undertook a 65 mile bicycle ride, extending over about 12 hours and including a considerable amount of hill-climbing. The data covering this period are given in Table II. A consideration of the results from these widely differing types of activity, especially as regards the changes in blood sugar, suggested the possibility that the human organism differs in its reaction to short strenuous effort and to long tedious work. Accordingly it was decided to divide the muscular work into two kinds, a short, strenuous period of activity, and an easier, but longer period. For the first type rapid stair-climbing was chosen—a mode of exercise often made use of in this connection. A double flight of stairs in the laboratory building, about 20 feet in height, was used, and the subjects ran up and down these stairs as rapidly as possible until nearly exhausted. The total time of the exercise did not exceed 15

minutes in any case. For the other type of exercise bicycle riding was chosen. Two subjects generally rode simultaneously and at such speed that they were in a distinct state of fatigue at

TABLE I.\*

Subject.	Total nitrogen.			Non-protein nitrogen per 100 cc.			Urea nitrogen per 100 cc.			Sugar per 100 cc.			Uric acid per 100 cc.		
	S	R	K	S	R	K	S	R	K	S	R	K	S†	R	K
	per cent	per cent	per cent	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.		mg.	mg.
I	3.37	3.55	3.35	49.0	39.6	45.3	19.2	14.8	20.7	118	86	110	<	2.3	
II	3.49	3.56	3.44	(45.6)	40.6	47.1	19.7	13.6	18.7	97	86	108	=	3.7	
III	3.57	3.62	3.40	49.4	44.9	46.7	20.0	15.0	18.9	127	131	131	>	5.1	
IV		3.72	3.51		46.4	50.0		18.6	23.2		92	111		7.6	

\* Of the horizontal rows numbered with Roman numerals I contains the results from the samples taken before the exercise; II, after the subject had run 100 yards; III, after running one mile; and IV, after 2½ hours subsequent rest. The vertical columns contain the results belonging to each of the three subjects, S, R, and K.

† The results in this column are only qualitative. The samples were not compared in the colorimeter, but II showed distinctly more uric acid than I, and III distinctly more than II.

TABLE II.\*

Subject.	Time.	Total N.	Non-protein N per 100 cc.	Urea N per 100 cc.	Uric acid per 100 cc.	Sugar per 100 cc.
		per cent	mg.	mg.	mg.	mg.
S	Before.	3.33	39.7	17.5	4.5	115
	After.	3.63	50.2	25.1	12.5	79
R	Before.	3.40	39.6	22.0	2(?)	106
	After.	3.48	43.9	29.3	3.7	100

\* Showing results before and after a 65 mile bicycle ride, occupying 12 hours. A small amount of food was taken during that time, principally four or five pieces of bread and butter, but the second sample was taken 5 hours after eating.

the end of from 2 to 3 hours. Most of the subjects were not accustomed to riding long distances.

It was decided to confine the attention to the changes in sugar, uric acid, non-protein and urea nitrogen, preformed and total

creatinine, and cholesterol. These were determined in the plasma as well as in the whole blood, whence an insight was obtained into the changes in the distribution of these materials between plasma and corpuscles. Total nitrogen was determined in the preliminary experiments, but this was not extended into the major portion of the work. In addition to these chemical determinations it was considered important to note the changes in certain physical properties of the blood, especially such as would throw light upon any possible variations in the blood volume. A direct determination of the changes in blood volume was originally undertaken with dogs, but was necessarily given up when the work was carried over to human subjects.

The experiments were generally carried out in the late afternoon, not closer than 3 to 4 hours from the noon meal. For collecting the blood samples a paraffined 50 cc. flask was used, fitted with a two-hole stopper carrying two right-angled glass tubes about 2 inches in length. One of these was connected with a piece of heavy rubber tubing through which the operator could exert suction at will; the other tube was paraffined internally and connected by a short piece of rubber tubing to a 20 gauge needle. A small amount of powdered potassium oxalate was introduced into the flask and 40 to 50 cc. of blood were drawn from an arm vein. A preliminary, or control, sample was taken in this way and another one, similarly, after the exercise.

### *Methods.*

*Chemical Determinations.*—For the determination of sugar, uric acid, non-protein nitrogen, urea, and creatinine, both preformed and total, the general procedure of Folin and Wu (1) was adopted because of its simplicity, reasonable accuracy, and economy of material. The proteins were precipitated both in plasma and whole blood in exactly the same manner, and 15 cc. of whole blood and 10 cc. of plasma yielded ample protein-free filtrate in each case to make determinations of sugar in duplicate or triplicate, urea and non-protein nitrogen in triplicate, total creatinine in duplicate, and preformed creatinine and uric acid in single determinations. Because of the uncertainty in preparing and keeping active urease solutions and because of the large number of samples urea was determined by the autoclave

modification, *i.e.* autoclaving at 150°C. for 10 minutes, in which case the total creatinine determinations could be started at the same time.

One more or less important departure from the original Folin-Wu method was made in the case of non-protein nitrogen. Largely as a matter of personal preference, but also because of some difficulty with cloudy solutions and refractory precipitates after digestion with the phosphoric-sulfuric mixture, non-protein nitrogen was determined as follows:

To 5 cc. of the protein-free filtrate in a large Pyrex test-tube about 0.5 gm. of potassium sulfate was added, one or two small pieces of broken porcelain, and 1 cc. of a 1:1 solution of sulfuric acid containing 1 gm. of copper sulfate per 100 cc. The solution was then evaporated to fumes of  $\text{H}_2\text{SO}_4$ , the tube covered with a 2 cm. watch-glass, and digested slowly for 5 minutes after the solution had become clear and bluish. It was then cooled and diluted to about 25 cc. After the addition of 5 cc. of 20 per cent NaOH, a drop of paraffin oil, and a little finely granulated zinc, the ammonia was distilled over into 5 cc. of twentieth normal HCl in a test-tube graduated at 50 cc. and Nesslerized as usual with 5 cc. of the reagent as prepared by Folin and Wu. For the distillation, the test-tube was fitted with a two-hole rubber stopper carrying in one hole an outlet tube to a 6 inch, three-bulb condenser, and in the other hole a 5 cc. pipette closed with a short piece of rubber tubing and a pinch-cock, which served as a means of introducing the NaOH after the test-tube was in position. The solution in the tube was gradually brought to boiling with a microburner and the air slowly expelled from the apparatus. The distillation was carried on for 4 minutes after the condenser began to deliver. Toward the end the apparatus was allowed to cool slightly and the acid to suck back for a short distance. The pinch-cock, closing the pipette in the distillation tube, was then opened for a moment to draw back into the tube any traces of ammonia which might have been trapped within the pipette. Distillation was then continued for a few moments longer before stopping and washing.

Although this method is somewhat longer than the direct Nesslerization, it is easily possible to distil ten to twelve samples in 1½ hours. With ordinary care there is no loss during distil-



lation; the method recovers ammonia quantitatively and is highly satisfactory. A blank determination of the reagents is of course necessary.

The same distilling apparatus above described was used for urea. For receiving the distillate the Folin blood sugar tubes are excellently adapted, being graduated at 25 cc. and having a constriction in the neck at the proper place to prevent any loss of ammonia by too rapid bubbling during the early stages of the distillation. . . Trouble with excessive frothing is generally traceable to the quality of paraffin oil used, and finely granulated zinc is preferable to quartz or porcelain to prevent bumping.

Cholesterol was determined by the method of Myers and Wardell (2), this procedure being found most satisfactory after trying out several of the colorimetric methods now in use. The only important modification made was the use of a standard solution of pure cholesterol in chloroform for the colorimetric comparison, in place of naphthol green B recommended by the authors of the method.

*Specific Gravity.*—The Hammerschlag method was first tried but was abandoned in favor of the Westphal balance, since ample material was available. For this purpose a round glass plummet was made having a volume of about 2 cc. This was suspended on a silk thread from the beam of the analytical balance, and by determining its weight in air, water, and blood, the specific gravity was calculated in the usual manner.

*Viscosity.*—A capillary pipette was made which delivered its contents of blood in 40 to 60 seconds. A fairly rapid delivery was advisable to avoid the settling of the corpuscles. This pipette was carefully calibrated with water and its time-of-delivery curve determined over a 10° range of temperature. Conditions did not permit of a very accurate temperature control of the blood, but it was found sufficiently accurate to use the average of the blood temperatures before and after the determination. It was ascertained that a change of 2 or 3° made practically no difference in the delivery time for blood, and the temperature regulation was well within these limits. For the measurement of the plasma viscosity the ordinary type of capillary viscosimeter was used, immersed in a constant temperature bath. The delivery time for plasma was about 2 minutes. All the data for viscosity are relative to water.

*Hemoglobin.*—The method of Cohen and Smith (3) was modified by pipetting 1 cc. of blood into about 200 cc. of water in a 250 cc. graduated flask. Then 25 cc. of normal HCl were added and the contents made up to the mark with water. Since only the relative, and not the absolute, amounts of hemoglobin were of interest two samples of blood were treated in this way, one taken before and one after the exercise, and the resulting colors of the acid hematin simply compared against each other in the colorimeter to obtain the "hemoglobin ratio," which is calculated on the assumption that the blood had a content of 100 per cent before the exercise.

*Relative Corpuscle Volume.*—The relative volume of corpuscles was determined in the customary way by the hematocrit method. The suggestions of Sundstroem and Bloor (4) in regard to the construction of hematocrit tubes, were more or less closely followed. A number of capillary tubes were cut about 11 cm. in length and ground flat at one end with emery. These were all filled from the same sample of blood, centrifuged for the same time, and those which indicated concordant corpuscle volumes accepted for use. A more rigid method of checking the regularity of bore was used in some cases, as follows: A short column of mercury was drawn up into a tube and the length of this mercury column carefully measured as it was moved to different positions within the capillary. If little or no variation in the length of the mercury column was observed the bore of the tube was considered sufficiently regular. When it was possible the same capillary was used for the second blood sample as for the first and centrifuged in each case for the same time at the same speed (1 hour at 2,000 R.P.M.). In other cases two capillaries, calibrated or checked against each other, were used to contain blood from the two samples, respectively, and centrifuged simultaneously for 1 hour at 2,000 revolutions per minute, at the end of which time the length of column was constant. Therefore, the comparative nature of these results is practically certain.

*Plasma-Corpuscle Relations.*—The concentrations of the various substances in the corpuscles were calculated in the usual manner from the plasma and whole blood concentrations. It must be kept in mind that the determination of corpuscle concentrations by difference in this way leaves considerable opportunity for the

accumulation of errors in the corpuscle results, in which there is consequently less certainty than in the directly determined values for plasma.

#### DISCUSSION.

A consideration of the data in Table I, which are more or less fragmentary, points out certain preliminary facts.

Total nitrogen exhibited a slight tendency to rise during short exercise, which apparently continued for some time after. Non-protein and urea nitrogen underwent no regular change excepting a possible slight increase after the exercise was over. These changes are in accordance with the heretofore observed tendency for the nitrogen elimination to increase *after* work.

Sugar was increased considerably by an amount of exercise represented by a mile run but returned to normal within  $2\frac{1}{2}$  hours. Uric acid was noticeably increased by short exercise and continued to increase for some time after. This also agrees with the general finding in regard to uric acid eliminated in the urine.

Table II, representing a much longer work period, presents a somewhat different picture. Total nitrogen and non-protein nitrogen, as well as urea, were increased. The concentration of uric acid rose considerably, but the longer exercise caused a drop in the blood sugar.

The more exhaustive investigation, represented in Tables III and IV, substantiates these preliminary conclusions and goes somewhat further. In order to facilitate comparison of the effects of the two kinds of exercise the average results in the two cases are brought together in Table V.

Let us consider each factor separately.

#### *Sugar.*

The shorter exercise period, which represented an expenditure, on the average, of about 58,000 foot pounds of energy in 10 minutes, resulted in an increase in whole blood sugar in seventeen cases out of eighteen. This increase averaged 0.036 per cent. The one instance of decrease was in the case of a subject who exhibited at another time an increase of about the same amount. It may possibly be significant that this subject, a boxer in training, was in the best physical condition of any who participated.

TABLE III.  
Short Period of Exercise.

No.	Subject.	Sugar.				Uric acid.				Urea nitrogen.				Non-protein nitrogen.		
		Whole blood.	Plasma.	Corpuscles.	Whole blood.	Plasma.	Corpuscles.	Whole blood.	Plasma.	Corpuscles.	Plasma.	Whole blood.	Corpuscles.	Plasma.	Whole blood.	Corpuscles.
1	♂NWR	110	96	129								43.3	38.1	37.4	50.7	50.6
		178	178	178								43.1				
1'	♂NWR	86	75	100	3.5	4.3		15.0	16.7	12.9	16.7	35.0	27.6	44.3		
		143	142	144				16.7	19.2	13.6		35.1	31.7	39.1		
1''	♂NWR	104	97	113	3.2	4.2	1.9									
		127	121	134	3.6	5.0										
2	♀ACA	94	98	88	6.6	6.5										
		162	160	165												
2'	♀ACA	100	92	112	2.9	2.9	2.9	14.4	14.2	14.7	14.6	29.6	24.0	24.2	38.0	39.1
		129	121	140	2.6	4.6	0	14.7		14.8		30.5				
3	♀BBR	87	89	85	4.5	5.4		16.9	16.6			36.0				
		125	126	124								37.2				
4	♂KDG	95	86	96	5.9	5.9		27.9	29.0	26.6	28.7	49.4	46.0	46.6	53.5	51.0
		131	124	138				28.2		27.7		48.8				
5	♀MEC	90			3.0	3.7	2.0	32.1	3.1			39.2				
		144				4.0	1.5	25.2				35.3				

6	♂ELG	94	81	113	5.0	5.7		21.8	21.9	21.7	41.7	32.5	55.0
		145	136	151				21.8	20.7	23.1	41.8	31.0	55.3
7	♂FAC	106	110	101	3.6	3.75	3.4	17.2	16.5	18.0	33.4		
		167	168	166	4.0	5.1	2.8	16.8	17.3	16.2	32.2		
8	♀MEG	79			3.5	3.8		21.3					
		132						21.3					
9	♂WGB	98	89	112	5.6	5.75	5.4	24.5	25.8	22.9	47.9	37.4	61.1
		112	110	115	6.0	6.3	5.6	22.9	26.4	18.3	48.9	37.9	63.5
9'	♂WGB	114	114	114		5.0		28.5	31.7	24.3			
		102	93	113				28.4	32.0	23.9			
10	♀EEA				2.6	1.4	4.5	13.9	14.7	12.7			
					2.8	1.65	4.5	13.8	15.5	11.3			
10'	♀EEA	94	87	104	3.1	3.5	2.5				27.1	19.4	38.0
		118	107	131		3.2	1.6				27.8	19.6	37.6
11	♂ABS	78	80	76		4.1		16.5	17.0	16.0	43.0		
		88	84	92			6.3	16.1	17.2	15.2	42.4		
12	♂MWG	97	98	96	2.9	2.6	3.3	18.6	18.5	18.7		29.5	29.6
		132	130	135	3.3	5.3	1.0	19.5	20.6	18.2			
13	♂EDB	95	91	99	5.4	6.7	4.1				40.0	33.8	46.1
		116	111	121	5.8	7.1	4.6				40.0	34.2	45.5

TABLE III—Continued.

No.	Subject.	Sugar.			Uric acid.			Urea nitrogen.			Non-protein nitrogen.		
		Whole blood.	Plasma.	Corpuscles.	Whole blood.	Plasma.	Corpuscles.	Whole blood.	Plasma.	Corpuscles.	Whole blood.	Plasma.	Corpuscles.
14	♀ GK	86	81	94		3.6		32.5	27.4		32.5	27.9	38.2
		102	101	103		4.0							
17	♂ FSE				4.45	6.4	2.1						
						6.85	1.9						
					4.65								
18	♂ CEH				5.4	6.4	3.9						
					5.75	7.7	3.3						
Averages....		95	91	102	4.2	4.3	3.4	20.6	20.6	18.8	38.3	31.4	47.5
		131	126	134	4.5	5.2	2.6	21.2	20.2	18.2	38.1	31.7	46.7

Subject No.	Preformed creatinine.				Total creatinine.			Specific gravity.	Relative viscosity.		Relative corpuscle volume.	Hemo-globin ratio.
	Whole blood.	Plasma.	Corpuscles.		Whole blood.	Plasma.	Corpuscles.		Whole blood.	Plasma.		
1	1.1	1.1	1.1								42	
1'	1.3	1.3	1.3						4.90	5.22	44.4	43
1''	1.3	1.3	1.3	5.1	2.8	2.8	8.0	1.054	4.32		44.6	
2	1.4	1.3	1.5	5.1	5.1	2.8	7.8	1.054	4.31		43.9	100
											45.8	102
2											38.5	
2'	1.2	1.1	1.3	5.9	3.2	3.2	10.0	1.055	4.18		40.0	
	1.2	1.2	1.25	7.0	7.0	3.6	11.6	1.060	4.39		42.3	
3									5.0	5.4	37.6	38.4
4	1.4	1.4							5.38	5.71	45.5	50.0
5	1.2	1.3							4.85	5.23	40.7	40.1

TABLE III—*Concluded.*

Subject No.	Preformed creatinine.			Total creatinine.			Specific gravity.	Relative viscosity.		Relative corpuscle volume.	Hemo-globin ratio.
	Whole blood.	Plasma.	Corpuscles.	Whole blood.	Plasma.	Corpuscles.		Whole blood.	Plasma.		
6	1.3	1.5						4.61	5.11	40.9	44.4
7	1.3	1.4						4.81	5.35	45.1	47.7
8	1.3	1.35		5.4			1.058	4.79	4.91		
9	1.5	1.5	1.5		6.0		1.060				
9'	1.3	1.3	1.3	5.1	3.0	7.9	1.056	4.63	1.64	44.4	43.3
10	1.2	1.15	1.25	4.8	3.2	7.5	1.058	4.05	4.63	42.4	44.4
10	1.35	1.25	1.5	5.2	3.3	8.0		4.05	4.35	39.2	40.1
10'	1.2	1.25	1.15	5.3	2.5	9.3	1.0555	4.44	1.53	41.4	100
10'	1.2	1.2	1.2	5.7	2.7	9.3	1.061	5.02	1.68	45.5	107
11	1.35	1.3	1.4	5.7	2.6	8.9		4.44	1.45	49.5	100
11	1.4	1.35	1.45	5.6	3.1	7.8		5.14	1.55	53.0	105



12	1.05	1.0	1.1	1.1				1.056	4.31	43.5	47.0	
	1.15		1.1	1.2				1.061	5.55			
13		1.2	1.2	5.9	3.0	8.8		1.063	5.00	50.3	100	102
				6.4	3.1	9.5		1.064	5.30	1.67	51.3	
14				4.8	3.1	7.4		1.053	3.60	39.8	100	108
				5.3	3.1	8.8		1.058	4.28	1.57	44.5	
17										45.5	44.6	
18										1.61	40.5	
										1.69	43.8	
Averages...	1.26	1.22	1.30	5.3	2.9	8.5		1.056	4.59	42.8	100	105
	1.33	1.26	1.36	5.7	3.1	8.7		1.060	5.00	1.64	44.7	

\* Showing the effect of short, intense exercise upon certain blood constituents. In each column the upper, left-hand number applies to the sample taken before the exercise, while the lower, right-hand one applies to the sample taken afterwards. The exercise consisted of running rapidly up a flight of stairs until nearly exhausted. The work varied from 30,000 to 100,000 foot pounds in an average time of 10 to 15 minutes. All values are expressed in mg. per 100 cc. of blood, except in cases otherwise apparent.

TABLE IV.  
*Long Period of Exercise.*

No.	Subject.	Sugar.				Uric acid.				Urea nitrogen.				Non-protein nitrogen.			
		Whole blood.	Plasma.	Corpuscles.	Whole blood.	Plasma.	Corpuscles.	Whole blood.	Plasma.	Whole blood.	Corpuscles.	Plasma.	Corpuscles.	Whole blood.	Plasma.	Corpuscles.	Corpuscles.
1	♂NWR	96	95	46	95	2.7	2.8	2.6	18.2	20.4	15.0	37.8	26.4	43.3	28.6	54.4	61.1
1'	♂NWR	93	84	104	113	4.0	4.8	3.0	15.3	16.1	14.3	32.3	22.1	36.6	24.6	45.3	50.6
11	♂ABS	92	79	106	101	7.3	8.3	6.2	18.8	18.8	18.8	44.9	30.3	38.9	28.1	60.7	51.9
13	♂EDB	98	104	91	112	5.6	7.7	3.2	20.5	22.2	18.6	45.8	28.3	46.3	32.3	65.8	61.8
6	♂ELG	102	97	109	109	5.6	7.3	3.0	17.3	19.7	13.7	37.1	24.7	38.2	25.3	55.8	56.7
15	♂WAC	117	115	120	125	4.0	3.1	5.1	18.8	17.8	20.1	43.8	26.5	49.6	28.7	65.4	75.6
14	♀GK	100	94	109	149	2.2	2.7	1.4	21.4	26.6	(13.4)	36.1	28.4	46.6	34.7	47.9	63.7
3	♀BBR	88	91	84	87	3.0	3.9	1.7	21.2	21.0	21.5	35.7	29.5	40.5	33.3	44.5	50.4
16	♀IGM	105	104	107	106	2.0	2.6	1.0	21.0	21.5	20.2	33.6	29.9	35.4	31.5	39.8	42.0
Averages .....		99	94	81	111	4.0	4.9	2.9	19.2	20.5	17.4	38.6	27.3	41.7	29.7	53.1	57.1

Subject No.	Preformed Creatinine.			Total Creatinine.			Specific gravity.	Relative viscosity.		Relative corpuscle volume.	Hemo-globin ratio.
	Whole blood.	Plasma.	Corpuscles.	Whole blood.	Plasma.	Corpuscles.		Whole blood.	Plasma.		
1	1.3 1.4	1.3 1.35	1.3 1.45	5.2 5.4	2.8 2.8	8.7 8.5	1.058 1.062	4.71 5.50	1.49 1.54	40.8 45.3	100 101
1'	1.2 1.3	1.1 1.2	1.3 1.4	4.4 4.5	2.05 2.05	7.4 7.4	1.061 1.064	4.83 5.64		43.9 45.5	
11	1.2 1.2	1.0 1.0	1.4 1.4	4.7 4.6	2.3 2.2	7.3 7.5	1.060 1.060	4.88 4.93		48.0 45.5	
13	1.2 1.2	1.1 1.1	1.3 1.3	4.9 4.8	2.1 2.1	8.1 7.8	1.062 1.063	5.93 5.42		46.8 47.5	100 100
6	1.1 1.1	1.0 1.0	1.2 1.2	4.1 4.3	2.3 2.4	6.8 7.0	1.059 1.059	4.64 4.64		39.9 41.1	
15	1.3 1.4	1.3 1.3	1.3 1.5	4.7 5.2	2.7 3.5	7.2 7.3	1.058 1.059	4.61 5.03		44.4 44.6	100 100
14	1.4 1.5	1.3 1.3	1.55 1.8	5.5 6.0	2.8 3.2	9.6 10.0	1.055 1.058	3.95 4.28	1.47 1.48	39.5 41.0	100 103
3	1.1 1.2	1.0 1.1	1.2 1.3	5.0 5.3	2.4 2.4	8.7 9.3	1.058 1.059	5.10 5.46	1.64 1.63	41.2 42.0	100 105
16	1.1 1.1	1.1 1.1	1.1 1.1	4.1 4.2	2.3 2.4	7.1 7.3	1.051 1.055	3.58 4.13	1.58 1.62	37.3 37.0	100 107
Averages ....	1.21 1.27	1.13 1.16	1.29 1.38	4.7 4.9	2.4 2.6	7.9 8.0	1.058 1.060	4.69 5.01	1.54 1.57	42.4 44.4	100 103

\* Showing the effect of long, moderate exercise (2 to 3 hours of fairly rapid bicycle riding) upon certain blood constituents. As in Table III, the upper left-hand numbers in each column apply to the first sample and the lower right-hand ones to the second.

TABLE V.  
*Comparison of Averages.*

Period.	Sugar.				Uric acid.				Urea nitrogen.			
	Whole blood.	Plasma.	Corpuscles.	$\frac{\text{Plasma}}{\text{Corpuscles}}$	Whole blood.	Plasma.	Corpuscles.	$\frac{\text{Plasma}}{\text{Corpuscles}}$	Whole blood.	Plasma.	Corpuscles.	$\frac{\text{Plasma}}{\text{Corpuscles}}$
Short.	95	91	102	0.9	4.2	4.3	3.4	1.3	20.6	20.6	18.8	1.1
	131	126	134	0.9	4.5	5.2	2.6	2.0	20.2	21.2	18.2	1.2
Long.	99	96	103	0.9	4.0	4.8	2.9	1.7	19.2	20.5	17.4	1.2
	94	81	111	0.7	4.9	6.3	3.1	2.0	20.9	21.7	19.9	1.1
Non-protein nitrogen.				Preformed creatinine.				Total creatinine.				
Short.	Whole blood.	Plasma.	Corpuscles.	$\frac{\text{Plasma}}{\text{Corpuscles}}$	Whole blood.	Plasma.	Corpuscles.	$\frac{\text{Plasma}}{\text{Corpuscles}}$	Whole blood.	Plasma.	Corpuscles.	$\frac{\text{Plasma}}{\text{Corpuscles}}$
	38.3	31.4	47.5	0.7	1.26	1.22	1.30	0.9	5.3	2.9	8.5	0.35
	38.1	31.7	46.7	0.7	1.33	1.26	1.36	0.9	5.7	3.1	8.7	0.35
Long.	38.6	27.3	53.1	0.5	1.21	1.13	1.29	0.9	4.7	2.4	7.9	0.3
	41.7	29.7	57.1	0.5	1.27	1.16	1.38	0.85	4.9	2.6	8.0	0.3

	Cholestrol.				Specific gravity.	Viscosity.		Relative corpuscle volume.	Hemoglobin ratio.
	Whole blood.	Plasma.	Corpuscles.	$\frac{\text{Plasma}}{\text{Corpuscles}}$		Whole blood.	Plasma.		
Short.	164	148	180	0.8	1.056	4.59	1.56	42.8	100
	155	155	156	1.0	1.060	5.00	1.64	44.7	105
Long.	161	157	165	0.9	1.058	4.69	1.54	42.4	100
	154	152	161	0.9	1.060	5.01	1.57	44.4	103

The increase in plasma and corpuscle sugar was of the same order of magnitude, and the exercise accordingly had no effect upon the distribution of sugar between the two fractions.

It will immediately be observed that the distribution of sugar in all these experiments is different from the distribution relation now generally accepted as correct. The consistent fact that the sugar in the corpuscles proved to be greater in amount than that in the plasma was the subject of much concern. The recent work of Ege (5) and others seems to have shown conclusively that the concentration of sugar in the corpuscles is about three-fourths of that in the plasma. Wishart (6) insists that results showing as much sugar in the corpuscles as in the plasma are due to faulty technique. Falta and Richter-Quittner (7) have even presented results indicating that the corpuscles normally contain no sugar whatever, but their work does not seem to have been generally accepted as yet. Thus far, however, no distribution data appear to have been given on the basis of the Folin-Wu method, and the reason for the discrepancy in this respect between the latter and other methods is being further investigated in this laboratory. It may be pointed out that the normal, resting value for whole blood is no higher than the commonly accepted average, and there seems to be no explainable reason why the plasma values should be so low. That an incomplete removal of proteins in whole blood did not result in these cases from too low an acidity at least, has been demonstrated, since blood sugar values were no lower when a distinct excess of sulfuric acid had been added.

Whatever the true explanation of the apparent discrepancy it will not affect the comparative nature of the values given in the tables.

When we turn to the longer period we find only one case out of nine in which there was an increase of more than 0.002 per cent, but on the contrary an average *decrease* of 0.005 per cent in the nine cases observed. The decrease was much more pronounced in the plasma, amounting to 0.015 per cent on the average, as a result of which the calculated sugar in the corpuscles increased 0.008 per cent. In only one case did the corpuscle sugar drop as much as 0.005 per cent, although the decrease in the plasma was often many times this. In fact there seems to

have been a distinct tendency for the corpuscles to resist a decrease in sugar content, and this is also observed in the one case of decrease in Table III.

In other words, the longer exertion changed the distribution coefficient for sugar slightly, with the result that, on the average, there was relatively more sugar in the corpuscles after the exercise than before. This conclusion must be taken advisedly, however, until the matter of normal distribution of sugar, using the Folin-Wu method, has been cleared up.

#### *Uric Acid.*

Both the short and the long exercise caused an increase in uric acid which was greater, on the average, in the plasma than in the whole blood, so that in many cases the calculated uric acid in the corpuscles actually decreased. This is the most distinct effect upon distribution which was observed in any of the materials, there being no doubt that there was relatively less uric acid in the corpuscles after exercise than before.

The further increase in uric acid after the completion of the exercise has been noted in the one case in Table III. Unfortunately it was impossible to follow this subsequent change in the rest of the subjects, but a more intensive investigation of this point will be made later.

#### *Urea and Non-Protein Nitrogen.*

The short period of exercise had no particular effect upon either urea or non-protein nitrogen, nor upon their distribution between corpuscles and plasma. Not only do the averages show close agreement but there are few wide variations in the individual results. These determinations were made in triplicate and each value generally represents the average of three results. The urea includes, as usual, the ammonia nitrogen.

The longer period of exercise apparently had a very slight augmenting effect upon the urea and a somewhat greater one upon the non-protein nitrogen. There was no change in the distribution coefficient of either urea or non-protein nitrogen, however.

*Preformed and Total Creatinine.*

The values for preformed creatinine remained remarkably constant in both periods of exercise. This is no more than would be expected from the repeatedly observed constancy of creatinine as a blood constituent. There seemed to be a very slight tendency to increase, however, since a number of cases showed a noticeable rise, but no instance of a fall was observed. This increase amounted to less than 0.1 mg. on the average and was equally distributed between corpuscles and plasma.

Total creatinine was slightly increased, on the average, in both periods of exercise, although the distribution coefficient remained unchanged. The increase was not invariable, however; in several cases there was no observable change but in only two was there even a slight decrease.

The colorimetric comparison in the creatinine determinations is by far the most difficult of any, giving a certain element of uncertainty to small differences. The effect of exercise upon the preformed and total creatinine of the blood must therefore be considered more or less doubtful.

*Number of Red Corpuscles.*

Along with the other determinations red blood counts were made on the first thirteen subjects in the short period. The time consumed was so considerable and the results themselves so uncertain and variable in unskilled hands that, after establishing the fact that an actual increase occurred, the counts were not made beyond this point. Though the individual results are not given in the table the average of 13 sets of determinations showed an increase from 5.36 million to 5.55 million per c.mm. This increase, though small, is in fair agreement with those reported by Hawk (8) and by Schneider and Havens (9) over relatively long periods of exercise.

*Specific Gravity, Corpuscle Volume, and Hemoglobin.*

Except in three cases in which no change was observed the specific gravity increased invariably in both periods of exercise. This increase amounted, on the average, to 0.004 and 0.002 in the two periods.



With very few exceptions the relative volume of the corpuscles increased noticeably.

The relative hemoglobin values, or "hemoglobin ratios," showed a slight increase in most of the cases observed. This increase averaged 3 to 5 per cent.

These changes in specific gravity, corpuscle volume, and hemoglobin content are undoubtedly to some extent attributable to increases in the number of corpuscles. An increase in the corpuscle volume has also been shown to result from the higher carbon dioxide content of the blood.

#### *Viscosity.*

With very few exceptions the viscosity of the whole blood increased considerably in both periods. The reason for this increase has been pointed out by Adam (10), who has shown that the blood viscosity is dependent not only upon the concentration of the blood and the number of corpuscles, but especially upon the carbon dioxide content. Since the latter fact was not fully appreciated until after this work was nearly completed no careful precautions were taken to make the viscosity measurements under similar conditions of carbon dioxide tension. In all cases the blood was mixed sufficiently to prevent settling of the corpuscles, but not shaken enough to result in the loss of much carbon dioxide. The values are, therefore, purely qualitative.

Adam has shown that the viscosity of the plasma is also affected by the carbon dioxide content and from his results it would seem that the slight increases in the plasma viscosity found in this work may be traced almost wholly to that source, since the plasma was not arterialized by shaking or passage of oxygen.

#### *Cholesterol.*

The data on cholesterol are collected separately in Table VI. The relative corpuscle volumes are not included, since most of the subjects are the same as those in Tables III and IV.

The changes in cholesterol were generally small and not thoroughly consistent. On the whole, however, there was a general tendency to decrease, more noticeable in the short period than in the long, to which the corpuscles contributed more than the plasma. Not only did the average show a slight decrease but in only three cases, in each period, was there an increase.

TABLE VI.  
Cholesterol per 100 cc.

Short period.				Long period.			
Subject.	Whole blood.	Plasma.	Corpuscles.	Subject.	Whole blood.	Plasma.	Corpuscles.
	mg.	mg.	mg.		mg.	mg.	mg.
1 ♂ NWR	162	155	171	1 ♂ NWR	159	167	149
	155	166	142		136	131	142
	225				163		
2 ♀ ACA	192			1' ♂ NWR	141		
	136	108	174		180	124	244
9 ♂ WGB	148	155	139	6 ♂ ELG	144	147	141
	155				143	142	145
11 ♂ ABS	155			11 ♂ ABS	150	156	143
	130				154	168	137
12 ♂ MWG	124			13 ♂ EDB	210	147	288
	180	155	205		150	162	132
13 ♂ EDB	140	174	108	14 ♀ GK	144	139	151
	165	175	150		147	166	123
14 ♀ GK	157	158	156	15 ♂ WAC	142	177	99
	161	158	166		169	161	180
16 ♀ IGM	167	161	177	3 ♀ BBR	169	177	158
	150	131	173		173	173	173
17 ♂ FSE	156	150	163	16 ♀ IGM	178	180	175
	157	141	180		153	158	146
18 ♂ CEH	138	119	162	5 ♀ MEC	125	149	89
	150	118	199		180	150	222
19 ♀ HCM	150	122	188	20 ♀ MJH	160	113	221
	150	155	142				
20 ♀ MJH	132	145	113				
	212	187	238				
21 ♂ RWS	204	196	213				
Averages . . . .	164	148	180		161	157	165
	155	155	156		154	152	161

*Considerations Bearing on Blood Volume.*

There are a number of considerations which substantiate the conclusion that the total blood volume underwent no considerable change during the course of these experiments: (1) The hemoglobin content did not increase much over 5 per cent in any case, which can to some extent at least be attributed to an increase in the number of red corpuscles which is known to have occurred. (2) Increases in specific gravity were slight and may also be explained as in (1). (3) The increase in the plasma viscosity, which would quickly reflect any considerable change in the blood concentration, was slight, and it is known that a considerable portion of this increase was due to a higher content of carbon dioxide. (4) The values for preformed creatinine, recognized as an especially constant blood constituent, showed very little increasing tendency. It is very likely that any considerable change in the blood volume would be here apparent.

The combined evidence from these different sources makes it practically conclusive that changes in blood volume cannot be regarded as important disturbing influences in the validity of the analytical results of these experiments.

## SUMMARY.

1. An investigation was undertaken on twenty-one human subjects to determine the changes produced by severe muscular exercise upon the following constituents of blood and plasma: Non-protein nitrogen, urea, sugar, uric acid, preformed and total creatinine, cholesterol, and hemoglobin, as well as specific gravity, viscosity, and the number and relative volume of corpuscles.

2. Two types of exercise were employed, representing short, strenuous effort and longer, more tedious work.

3. Short, strenuous exercise was invariably found to increase the blood sugar concentration both in plasma and corpuscles, while a longer period of exercise was generally accompanied by a drop in blood sugar, which was greater in the plasma than in the whole blood.

4. Both kinds of exercise were accompanied by a small increase in uric acid, of about the same order, which was greater in the plasma than in the whole blood.

5. Short, strenuous exercise had no effect upon urea or non-protein nitrogen, but longer work increased both slightly, in whole blood as well as plasma.

6. In both types of exercise the total creatinine increased very little, while the preformed creatinine underwent almost no change.

7. It is shown conclusively that there were no considerable changes in the total blood volume during the muscular exercise and that variations in the concentration of the blood are not, therefore, disturbing factors in the above conclusions.

8. Cholesterol was found to decrease very slightly, although results were not thoroughly consistent. The decrease seemed to be somewhat more noticeable in the corpuscles than in the plasma.

9. The specific gravity, hemoglobin, and the number and relative volume of corpuscles were found to increase during the periods of exercise. The viscosity of the whole blood was found to increase considerably and that of the plasma slightly.

10. Some incomplete data are given suggesting that total nitrogen is increased in the blood by exercise and that urea, non-protein nitrogen, and uric acid continue to increase for some time after a work period, while the sugar concentration, on the other hand, returns to normal within  $2\frac{1}{2}$  hours.

The author wishes to express his thanks to Professor R. E. Swain, at whose suggestion this work was undertaken, for many helpful suggestions; to the several students and others who volunteered as subjects; and to Miss Dorothy E. Bernard, who was responsible for the analyses of cholesterol.

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# THE JOURNAL

OF

# BIOLOGICAL CHEMISTRY

FOUNDED BY CHRISTIAN A. HERTER AND SUSTAINED IN PART BY THE CHRISTIAN A. HERTER  
MEMORIAL FUND

OFFICIAL ORGAN OF THE AMERICAN SOCIETY OF BIOLOGICAL CHEMISTS

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VOLUME XLVIII  
BALTIMORE  
1921

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THE JOURNAL OF BIOLOGICAL CHEMISTRY

PUBLISHED BY THE ROCKEFELLER INSTITUTE FOR MEDICAL RESEARCH FOR THE  
JOURNAL OF BIOLOGICAL CHEMISTRY, INC.

WAVERLY PRESS  
THE WILLIAMS & WILKINS COMPANY  
BALTIMORE, U. S. A.

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# METHODS FOR THE DIRECT QUANTITATIVE DETERMINATION OF SODIUM, POTASSIUM, CALCIUM, AND MAGNESIUM IN URINE AND STOOLS.

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(Received for publication, May 23, 1921.)

In the study of the inorganic metabolism of children it is frequently necessary to perform a large number of determinations of the various inorganic elements in urine and stools. The question of the amount of material available and the time required for a given determination becomes an important consideration. We have elsewhere reported simple methods for the quantitative estimation of sodium, potassium, calcium, and magnesium in serum (1, 2, and 3). It has been found possible to modify the sodium, potassium, and calcium methods so as to make them applicable to the acid extract of the partly ashed residue of urine and stools. For the determination of magnesium in urine and stools we have used the principle of alkalimetric titration of ammonium magnesium phosphate first suggested by Stolba (4). This procedure was subsequently modified by Mohr (5) and Kraus (6). Recently the same principle has been used by Bauzil (7), Angiolani (8), and Fiske (9) for the estimation of inorganic phosphates in urine. By the use of the methods described below a considerable saving in time and material is effected. The degree of accuracy of these methods is indicated in the tables.

## *Preparation of Material.*

*Stools.*—The fresh stool for a measured period is collected in a weighed porcelain dish. This is heated on the water bath until dry. 95 per cent alcohol is added and evaporated. This latter procedure is repeated, making two additions of alcohol in all. After thorough drying over the water bath the dish and contents

## 2 Inorganic Elements in Urine and Stools

are weighed and the weight of the air-dried stool is found. The fecal material is then ground to a fine powder and placed in a stoppered container. 2 gm. of this material are weighed in a platinum crucible and ashed for  $1\frac{1}{2}$  hours by the Stolte (10) method.<sup>1</sup> An ash-free filter paper (No. 40 Whatman, 11 cm. in diameter) is then washed by allowing 20 to 30 cc. of 0.5 N HCl to run through it. The platinum crucible containing the partly ashed stool is placed on the water bath and 10 cc. of 0.5 N HCl are added. After this has become hot it is transferred with a 10 cc. pipette and filtered through the washed filter paper into a 100 cc. flask. The procedure is repeated until the volume has been made up to 100 cc. The results obtained on this extract are identical with those found on a solution of completely ashed stool (Table VI).

*Urine.*—A measured quantity of urine (50 or 100 cc.) is evaporated in a platinum dish, ashed, and extracted in a similar manner to the stool. The extract is then made up to the original volume of the urine.

### *Sodium.*

The sodium is determined directly by precipitation as the pyroantimonate. As the precipitation must be carried out in an alkaline medium, calcium and any other elements present, which would form insoluble compounds such as tertiary calcium phosphate, must be removed, otherwise they would interfere with the gravimetric determination of the sodium pyroantimonate. This is accomplished by the following procedure. 15 to 20 cc. of the stool extract or 5 to 10 cc. of the urine extract are placed in a platinum dish and evaporated to dryness. The ash is transferred to a graduated centrifuge tube with 2.5 cc. of 0.5 N HCl. 3 cc. of a saturated solution of ammonium oxalate are added and the mixture is allowed to stand for 10 minutes. This precipitates practically all the calcium. Concentrated  $\text{NH}_4\text{OH}$  is then added to 7 cc., the contents are mixed and allowed to stand 45 minutes.

<sup>1</sup> The platinum dish which contains the material to be ashed is placed in a quartz dish 10 cm. in diameter and 6 cm. deep, in the bottom of which are placed several pieces of porcelain. The outer dish is gradually heated with a Meeker burner until no more fumes are given off when the flame is turned on full until the charred material is immobile. The large dish is then covered with a quartz plate and heating continued for  $1\frac{1}{2}$  hours.



Magnesium is thereby precipitated as ammonium magnesium phosphate. The sample is centrifuged for 5 minutes, after which 5 cc. of the supernatant fluid are placed in a platinum dish and evaporated to dryness. The dish is placed in the oven at 100°C. for a few minutes to thoroughly dry the residue in order to avoid spattering during the subsequent ashing. The sample is then ashed by the Stolte method for 15 to 30 minutes. This volatilizes all the ammonium salts. A small amount of ash remains in the dish. This dissolves readily in 2 cc. of 0.1 N HCl. A drop of phenolsulfonephthalein is added and the solution made just alkaline by the addition of 2 or 3 drops of 10 per cent KOH. This solution which contains all the sodium present in the original sample is now ready for the direct estimation of this element.

TABLE I.

*Determination of Sodium in Known Solutions of Inorganic Salts.\**

Amount of sample.	$\text{Na}_2\text{H}_2\text{Sb}_2\text{O}_7 + 6\text{H}_2\text{O}$ calculated.	$\text{Na}_2\text{H}_2\text{Sb}_2\text{O}_7 + 6\text{H}_2\text{O}$ found.	Error.
cc.	mg.	mg.	per cent
$\frac{5}{16}$ of 10	37.5	36.0	-4.2
$\frac{15}{16}$ " 10	37.5	38.1	+1.7
$\frac{5}{16}$ " 15	56.0	54.7	-2.3
$\frac{15}{16}$ " 15	56.0	56.2	+0.4
$\frac{5}{16}$ " 20	75.0	72.5	-3.3
$\frac{15}{16}$ " 20	75.0	72.8	-3.0

\* Composition of Solution Y.

NaCl.....	0.241 gm.
KCl.....	0.201 "
MgSO <sub>4</sub> + 7 H <sub>2</sub> O.....	0.130 "
Ca <sub>3</sub> (PO <sub>4</sub> ) <sub>2</sub> .....	0.330 "
CaCO <sub>3</sub> .....	0.500 "
0.5 N HCl to.....	200 cc.

To the sample, prepared as outlined above, are added 10 cc. of the potassium pyroantimonate reagent followed by 3 cc. of 95 per cent alcohol. The alcohol should be added, drop by drop, and the specimen stirred with a rubber-tipped rod. After standing 30 minutes, the precipitate is transferred to a weighed Gooch crucible and washed with 5 to 10 cc. of 30 per cent alcohol. The crucible is dried at 110°C. for 1 hour,<sup>2</sup> cooled in a desiccator for 30 minutes,

<sup>2</sup> The temperature is gradually raised to 110°C.

and weighed. The weight of the precipitate divided by 11.08 equals the number of mg. of sodium present in the sample.

The method of preparation of the potassium pyroantimonate reagent has been fully described in a former paper on the determination of sodium in serum (1). The details of the method of preparation of the Gooch crucibles, the precautions to be observed during the addition of the alcohol and the filtration and also the care of the platinum are fully outlined in the same paper.

The results given in Table I show that as little as 3 or 4 mg. of sodium may be quantitatively recovered from solutions containing relatively large amounts of calcium phosphate.

#### *Potassium.*

The potassium method is identical with that reported a short time ago by the authors for the estimation of this element in serum (2). The optimum amount of stool extract for the potassium determination is generally 1 cc. The concentration of this element in urine, however, is so high that it is necessary to take only 0.2 to 0.5 cc. The sample to be analyzed is placed in a graduated centrifuge tube and diluted with distilled water to 2 cc. The centrifuge tube should be previously cleaned with the use of a brush, washed out with strong cleaning fluid (commercial  $\text{H}_2\text{SO}_4$  and potassium dichromate), and then thoroughly rinsed with distilled water. If the tubes are not cleaned in this manner the precipitate will adhere to the sides and low results will be obtained. 1 cc. of the sodium cobalti-nitrite reagent is then slowly added, drop by drop. The contents of the tube are mixed and allowed to stand for  $\frac{1}{2}$  hour. The volume is made up to 5 cc. with water and the contents again mixed and the tube centrifuged for 7 minutes at about 1,300 revolutions per minute. The precipitate will then be found at the bottom of the tube. All but 0.2 to 0.3 cc. of the supernatant fluid is removed. This is accomplished by means of the following apparatus. Through one opening of a two-holed cork is inserted a glass tube by means of which a positive pressure can be made in the centrifuge tube. Through the other hole is placed a tube which reaches to about 3 or 4 mm. above the precipitate. The lower end of this tube is drawn out to a bore of about 1 mm. and curved so that the opening is directed upward. By

fitting the cork to the centrifuge tube and blowing through the first opening the supernatant fluid can be readily removed without disturbing the precipitate. 5 cc. of water are allowed to run down the side of the tube which is then gently agitated so that the added water is mixed thoroughly with the residual reagent. Care should be taken that the precipitate itself is disturbed as little as possible. This may be accomplished by holding the tube vertically and gently hitting the lower end with a circular motion. The brown fluid may be seen to rise and mix with the supernatant fluid. The tube is then centrifuged for 5 minutes. The procedure is repeated three times so that the precipitate is washed four times in all. The supernatant fluid from the last washing should be perfectly clear. After the removal of the fluid from the final washing the precipitate is ready to be titrated.

*Titration.*—An excess of 0.02 N potassium permanganate (generally 2 to 5 cc.) is added to the precipitate followed by 1 cc. of 4 N  $\text{H}_2\text{SO}_4$ . It is rather difficult to judge the amount of permanganate necessary to be added, but by carefully watching the tube while it is being heated, more can be added. The precipitate is thoroughly mixed with the permanganate and  $\text{H}_2\text{SO}_4$  by means of a glass rod and the tube is placed in the boiling water bath. At the end of 20 to 25 seconds the tube is examined and if the pink color of the permanganate has nearly disappeared, more permanganate is added from the micro-burette. In this way it is not difficult to find out how much permanganate is necessary to constitute an excess. At the end of 1 minute from the time the heating is begun, the solution should be of a perfectly clear pink color. If all the precipitate is not oxidized, the contents will be cloudy and the color will be seen to fade. Heating should then be continued until the solution is clear and pink. When the heating is continued too long, the contents again become cloudy and of a brownish color. If this is allowed to happen, the sample must be discarded as high results will be obtained. 2 cc. of 0.01 N sodium oxalate are promptly added and the contents mixed. If this is not sufficient to decolorize the permanganate, another 2 cc. should be immediately added. The excess of oxalate is then titrated with 0.02 N potassium permanganate delivered from a micro-burette, graduated in 0.02 cc., until a definite pink color is obtained which lasts for 1 minute.

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*Calculation.*—1 cc. of 0.01 N potassium permanganate will oxidize a quantity of potassium cobalti-nitrite corresponding to 0.071 mg. of potassium. Thus, if 2 cc. of 0.02 N potassium permanganate are originally added and 0.43 cc. of the same solution used in the final titration and 2 cc. of 0.01 N sodium oxalate are required to decolorize the sample after the first oxidation, then  $2.43 - 0.03$  (the amount of permanganate necessary to colorize the same quantity of water)  $\times 2$  (to convert 0.02 to 0.01 N)  $- 2.00$  (cc. of 0.01 N sodium oxalate added to decolorize the sample)  $\times 0.071 = 0.199$  mg. of K in sample.

The details of the preparation of the reagents have been given in a former paper on the determination of potassium in serum.<sup>3</sup>

TABLE II.

*Determination of Potassium in Known Solutions of Inorganic Salts Containing an Excess of Calcium Phosphate.\**

Amount of sample.	K present.	K found.	Error.
cc.	mg.	mg.	per cent
0.4	0.215	0.218	+1.4
0.5	0.269	0.274	+1.8
0.7	0.376	0.384	+2.1
1.0	0.538	0.538	$\pm 0.0$

\* Composition of Solution C.

$\text{Ca}_3(\text{PO}_4)_2$ .....	2.5820 gm.
KCl .....	0.2052 "
NaCl.....	0.2063 "
$\text{MgSO}_4 + 7 \text{H}_2\text{O}$ .....	0.6100 "
N HCl to.....	200 cc.

The results of the estimation of potassium by this method in a known solution of inorganic salts are given in Table II. It should be noted that even a large amount of phosphate does not affect the potassium determination.

### *Calcium.*

The calcium method is identical with that reported a short time ago by the authors for the estimation of this element in serum (3).

<sup>3</sup> Kramer and Tisdall (2), p. 343.

The concentration of calcium in the stool extract, however, is so high that dilution is necessary. 5 cc. of the extract are diluted to 50 cc. with distilled water. The optimum amount of this solution for the calcium determination is generally between 1 and 4 cc. The amount of the urine extract corresponding to 1 to 4 cc. of urine is also found to be quite satisfactory.

The sample (generally 2 cc.) is measured into a graduated centrifuge tube previously cleaned with commercial  $\text{H}_2\text{SO}_4$  and dichromate and the volume made up to 3 or 4 cc. with distilled water. A drop of phenolsulfonephthalein is added and 10 per cent  $\text{NH}_4\text{OH}$  (10 cc. concentrated  $\text{NH}_4\text{OH}$  in 90 cc. of  $\text{H}_2\text{O}$ ) until the solution is alkaline. Approximately  $\text{N}$   $\text{H}_2\text{SO}_4$  is added until the solution is just acid and any phosphates that may have been precipitated are redissolved. 1 cc. of approximately  $\text{N}$  oxalic acid is added followed by 1 cc. of a filtered saturated solution of sodium acetate which should be added drop by drop. The contents are mixed and allowed to stand for  $\frac{3}{4}$  hour when they are centrifuged for 10 minutes at about 1,300 revolutions per minute. This throws all the calcium oxalate precipitate to the bottom of the tube. All but 0.3 cc. of the supernatant fluid is removed by means of the apparatus described under the potassium method. The remaining fluid and the precipitate are mixed by tapping the tube. Enough 2 per cent ammonia (2 cc. of concentrated ammonia diluted to 100 cc.) is then added to bring the volume to 4 cc., care being taken to wash the sides of the centrifuge tube free from adherent oxalic acid. The tube is then centrifuged for 5 minutes. This procedure is repeated twice, thus making three washings in all. After the third washing the supernatant fluid is removed, the tube is shaken to suspend the precipitate, 2 cc. of approximately  $\text{N}$  sulfuric acid are added, and the tube is warmed in the boiling water bath for a few minutes and titrated with 0.01  $\text{N}$  potassium permanganate until a definite pink color persists for at least 1 minute when viewed under a good light against a white background. The strength of the permanganate solution is determined by titrating against 0.01  $\text{N}$  sodium oxalate (Sørensen).

*Calculation.*—The number of cc. of 0.01  $\text{N}$  potassium permanganate used (generally 0.5 to 2 cc.) — 0.02 cc. (the blank)  $\times$  0.2 = mg. of calcium in the sample.

*Preparation of Reagents.*—0.01  $\text{N}$  sodium oxalate (Sørensen) is

## 8 Inorganic Elements in Urine and Stools

the only reagent that must be quantitatively accurate. An 0.1  $\times$  sodium oxalate (Sörensen) is prepared in the usual way. 6.7 gm. of sodium oxalate (Sörensen) are dissolved in water. Solution is facilitated by the addition of 5 cc. of concentrated sulfuric acid and the volume made up to 1 liter. This is diluted ten times to make a 0.01  $\times$  solution. The former solution will keep indefinitely while the latter has been found still unchanged after the lapse of 2 months.

*Approximately  $\times$  Oxalic Acid.*—This is prepared by dissolving 63 gm. of oxalic acid (Kahlbaum or J. T. Baker, c. p., calcium-free) in a liter of water. The acid need be weighed only roughly.

*Approximately  $\times$  Sulfuric Acid.*—50 cc. of concentrated sulfuric acid (c. p.) are diluted with water to 1 liter.

TABLE III.

*Comparison of Calcium Determination on Solutions of Ash of Infants' Stools by McCrudden's Method and the Authors' Method.\**

Specimen.	McCrudden's method. Ca per 0.5 gm. stool.	Authors' method. Ca per 0.5 gm. stool.	Difference.
	<i>mg.</i>	<i>mg.</i>	<i>per cent</i>
I	31.17	30.09	-3.0
II	39.89	40.05	+0.4
III	35.11	34.50	-1.7
IV	53.16	51.18	-3.7

\* We are indebted to Dr. S. G. Ross for most of the calcium and magnesium determinations made by McCrudden's method.

*Saturated Sodium Acetate Solution.*—This solution is made by adding an excess of the salt to water and allowing it to stand over night. The supernatant fluid is then filtered. Sodium acetate (J. T. Baker, c. p.) does not contain calcium.

A comparison of the results obtained by this method and by the McCrudden method (11) on a solution of stool ash is given in Table III.

### *Magnesium.*

The principle used for the determination of magnesium is that originally advanced by Stolba (4) in 1877. The calcium is precipitated as the oxalate. The magnesium is then precipitated as

ammonium magnesium phosphate. An excess of HCl is added and the following reaction takes place:



The  $\text{H}_3\text{PO}_4$  is then titrated with 0.1 N NaOH to  $\text{NaH}_2\text{PO}_4$ , the pH of which is 4.4. The indicator used to detect the end-point is cochineal. This indicator changes from yellow to purple at pH 4.8. The error produced by titrating to this pH instead of pH 4.4 is small (9). 0.1 gm. molecules of  $\text{NH}_4\text{MgPO}_4$  when titrated with 0.3 gm. molecules of HCl yields 0.1 gm. molecules of  $\text{H}_3\text{PO}_4$ . When this is titrated back with 0.1 N NaOH to a pH of 4.8, one

TABLE IV.

*Determination of Magnesium in Samples of Solution A.\**

Solution A.	Titration reading. 0.1 N acid.	Magnesium found.	Magnesium present.	Error.
cc.	cc.	mg.	mg.	per cent
3	0.75	0.91	0.90	+1.1
5	1.20	1.45	1.50	-3.3
10	2.50	3.02	3.00	+0.7
20	5.10	6.17	6.00	+2.8

\* Composition of Solution A.

MgSO <sub>4</sub> containing Mg.....	0.030 gm.
CaCO <sub>3</sub> .....	1.154 "
Na <sub>2</sub> HPO <sub>4</sub> + 2 H <sub>2</sub> O.....	0.617 "
Concentrated HCl.....	5 cc.
H <sub>2</sub> O to.....	100 "

equivalent of H has been replaced by sodium, leaving two equivalents still united to  $\text{PO}_4$  as  $\text{NaH}_2\text{PO}_4$ . Therefore, two equivalents of H are equal to two equivalents of Mg; *i.e.*, 1 cc. of 0.1 N HCl = 1 cc. of 0.1 N Mg solution = 1.21 mg. of Mg.

The procedure is as follows: 25 to 50 cc. of the urine extract or 10 to 30 cc. of the stool extract are placed in a 100 cc. beaker. To this is added a drop of phenolsulfonephthalein and 10 per cent  $\text{NH}_4\text{OH}$  (10 cc. of concentrated  $\text{NH}_4\text{OH}$  in 90 cc. of  $\text{H}_2\text{O}$ ) until the solution is just alkaline. 4 N  $\text{H}_2\text{SO}_4$  is added until the solution is acid and all the phosphates are redissolved. 10 cc. of saturated ammonium oxalate are then added to the stool extract or 5 cc. to the urine extract, mixed, and allowed to stand for 15 minutes.

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This precipitates the calcium as the oxalate. 1 cc. of 10 per cent  $(\text{NH}_4)_2 \text{HPO}_4$  is added to insure an excess of phosphate followed by 5 cc. of concentrated  $\text{NH}_4\text{OH}$ . The mixture is thoroughly mixed, allowed to stand 1 hour, and then filtered through 9 cm. of

TABLE V.

*Comparison of Magnesium Determinations on Solutions of Ash of Infants' Stools by McCrudden's Method and the Authors' Method.*

Specimen.	McCrudden's method. Mg. in 0.5 gm. stool.	Authors' method. Mg. in 0.5 gm. stool.	Difference.
	<i>mg.</i>	<i>mg.</i>	<i>per cent</i>
I	2.84	2.88	+1.4
II	2.29	2.16	-5.7
III	2.78	2.70	-2.9
IV	3.55	3.54	-0.3

No. 40 Whatman filter paper. The precipitate is all transferred from the beaker by the use of a rubber-tipped rod and 10 per cent  $\text{NH}_4\text{OH}$ . The ammonia is then all removed from the filter paper by washing it four times with 30 per cent alcohol. The filter paper with the precipitate which includes the calcium oxalate

TABLE VI.

*Comparison of Calcium, Magnesium, Sodium, and Potassium Determinations on the Solutions of Stool Residues Which Were Ashed 1½ and 12 Hours.*

Specimen.	Inorganic element.	Ashed for 1½ hours.	Ashed for 12 hours.
		<i>gm. per 24 hrs.</i>	<i>mg. per 24 hrs.</i>
I	Ca	1.728	1.692
	Mg	0.115	0.115
	Na	0.514	0.510
	K	0.630	0.649
II	Ca	2.244	2.352
	Mg	0.104	0.108
	Na	0.035	0.037
	K	0.243	0.249
III	Ca	1.083	1.095
	Mg	0.081	0.084
	Na	0.146	0.145
	K	0.338	0.336



is transferred to a 100 cc. beaker, about 30 cc. of warm water are added, and the filter paper and precipitate mixed by the use of a glass rod. 3 drops of tincture of cochineal<sup>4</sup> are added and an excess of 0.1 N HCl (generally 5 cc.). After 5 minutes the mixture is titrated with 0.1 N NaOH delivered from a burette graduated in 0.05 cc. until the color changes from a light yellow to a purple. This end-point is very definite and a decided change in color is produced by 1 drop of 0.1 N NaOH. The presence of the filter paper and the large amount of calcium oxalate do not interfere with the interpretation of this end-point. The number of cc. of 0.1 N HCl added — the number of cc. of 0.1 N NaOH  $\times 1.21$  = the number of mg. of magnesium in the sample. An analysis of the magnesium content of a known solution of stool and urine salts is given in Table IV. A comparison of the results obtained by the above method and by McCrudden's method (11) is given in Table V.

Table VI shows that practically identical results are obtained on the acid extracts of partly ashed stools as on the acid solution of the completely ashed stools.

#### CONCLUSIONS.

1. Rapid methods for the determination of sodium, potassium, calcium, and magnesium in urine and stools, including a direct method for the determination of sodium in the presence of large quantities of other salts, particularly calcium phosphate, have been described.

2. The determination of potassium by means of sodium cobaltinitrite reagent has been used for the estimation of small amounts of this element in urine and stools. The error by this method is generally within 1 or 2 per cent.

3. The determination of calcium and magnesium has been compared with the results obtained by the standard McCrudden method. The deviation from the standard method has generally been within 3 per cent.

4. By means of these methods a considerable saving of time is effected and all the so called fixed alkali elements may be determined quantitatively in 50 cc. of urine or 2 gm. of dry stool.

<sup>4</sup> The tincture is made by digesting 1 part of crushed cochineal with 10 parts of 25 per cent alcohol.

## 12 Inorganic Elements in Urine and Stools

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## A RAPID METHOD FOR THE DETERMINATION OF HIPPURIC ACID IN URINE.\*

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(Received for publication, June 20, 1921.)

In making benzoate tests for renal efficiency we were confronted with the necessity for having a rapid and accurate method for the determination of hippuric acid in urine. The Folin-Flanders method which we have been using required more time than was thought necessary. By means of this method analyses could be made in 9 or 10 hours when necessary, but with the routine of teaching and other university work 24 hours were usually required. It was our object to devise a method which would conserve the accuracy of the Folin-Flanders (1) method, but one which could be completed within 2 or 3 hours and be as applicable for hospital routine work as are any of the other modern biochemical methods.

A careful review of the more recent methods for the determination of hippuric acid shows that at present there is only one method which fulfils the requirements of accuracy and simplicity. This is the method of Folin and Flanders. Two other methods which appeared at about the same time, Steenbock's (2) and Hryntschak's (3) methods meet the requirements of accuracy fairly well but are too tedious to compete with the Folin-Flanders method. Ito's (4) method appearing 4 years later is more complicated than those mentioned above and does not represent an advance in this field. Steenbock's and Hryntschak's procedures depend upon the isolation and weighing of benzoic acid, which are accompanied by slight losses, more in the latter method than in the former, and are necessary only in those cases in which benzoic acid cannot be directly titrated, as for instance, in the presence of other titratable

\*Acknowledgment is made to the Graduate School of the University of Minnesota for the purchase of a portion of the chemicals used in this work.

acids. Since there are no other acids present in the final extraction and titration stages of the Folin-Flanders method, titration in this case is not only easier to accomplish but more accurate.

Folin and Flanders proved that their method gave quantitative results with pure hippuric acid solutions which we have confirmed many times in the last few years. They did not compare their method, as applied to urine with any other procedure of analysis, nor as far as we can find, has any other investigator. They have assumed, however, that their method gives the most accurate results of any method devised up to that time. We have proved in the experiments which are directly to follow that the Folin-Flanders method does correctly estimate the amount of hippuric acid that can be extracted directly from urine by means of ethyl acetate.

*Experiment 1.*—0.561 gm. of pure sodium hippurate was dissolved in 100 cc. of water, 1 cc. of concentrated nitric acid added, and the mixture then extracted with ten 50 cc. portions of ethyl acetate, shaking exactly 2 minutes each time. The aqueous mixture left was then filtered, the filtrate evaporated to dryness over night on the steam bath with 10 cc. more of 5 per cent sodium hydroxide than that required for neutralization of the nitric acid present. The residue was then analyzed for any remaining hippuric acid by the Folin-Flanders method. The titration value was 0.07 cc., which is the ordinary blank of the method. The hippuric acid was completely extracted by this procedure.

100 cc. of urine, the hippuric acid titration value of which was 13.58 cc. of one-tenth normal sodium ethylate were acidified with 2 cc. of concentrated nitric acid and extracted with ten 50 cc. portions of ethyl acetate, shaking 2 minutes each time. The combined extracts were washed with two 200 cc. portions of the Folin-Flanders sodium chloride solution and then steam distilled until all of the ethyl acetate and approximately 300 cc. of water had passed over. The aqueous solution of hippuric acid remaining in the distilling flask was quantitatively transferred to a casserole and analyzed according to the Folin-Flanders method. The titration value was 13.43 cc. of one-tenth normal sodium ethylate, agreeing with the value obtained directly as well as duplicates can usually be obtained by this method.

#### *Experimental Methods of Analysis.*

Our problem resolved itself into increasing the speed of the hydrolysis of hippuric acid either by acids or alkalies and the effective oxidation of urinary pigments and other disturbing substances. Without going into the details of many experiments

carried out it may be stated that by using 15 gm. of solid sodium hydroxide in hydrolyzing the hippuric acid of 100 cc. of urine at the boiling point for 30 minutes and subsequently acidifying, extracting, and titrating, results were obtained that were, in one experiment, 22 per cent higher than the known titration value for this specimen of urine. It was also found that values from 10 to 33 per cent higher than those obtained by the Folin-Flanders method resulted when urine was boiled with an equal volume of a mixture of concentrated nitric and sulfuric acids for 30 minutes in a process that gave 100 per cent recovery when applied to solutions of pure hippuric acid. Oxidation of the urine with alkaline potassium permanganate after the plan of Hryntschak was tried and yielded such promising results that the details of one typical experiment are given below:

*Experiment 2.*—50 cc. of urine were boiled with 7.5 gm. of solid sodium hydroxide and 1.5 gm. of potassium permanganate for 30 minutes in a Kjeldahl flask with a rather closely fitting test-tube condenser in the neck. The flask was cooled and 50 cc. of concentrated nitric acid slowly poured down the side of the condenser. The brown mixture cleared up after boiling a few minutes, but this was continued for 30 minutes, then cooled and extracted as in the Folin-Flanders procedure using comparative amounts of the various materials; The titration value was 16.72 cc. of one-tenth normal sodium ethylate; by the regular Folin-Flanders method, 16.95 cc. In a series of 12 analyses made in this way it was found that values from 97 to 99 per cent of the Folin-Flanders figures could always be obtained when these were as large as 15 cc., but with lower values the error was sometimes as large as 25 per cent. This was believed to be due to the action of the potassium permanganate on the benzoic acid present as it was always most pronounced in the urines which were the most dilute and therefore containing less of the other substances to combine with the permanganate. It was difficult to estimate the correct amount of potassium permanganate to be added in each case and it frequently happened that 1.5 gm. were a greater amount than could be reduced beyond the manganate stage and 0.5 gm. portions of sodium bisulfite had to be added to complete the reduction. It was also found that if this method were applied to a pure solution of hippuric acid, allowing the potassium permanganate to act only 2 or 3 minutes before reducing it with sodium bisulfite that it was impossible to obtain more than 95 per cent of the theoretical amount. In Hryntschak's method the urine was boiled with 10 gm. of sodium hydroxide for 2.5 hours then 10 gm. of potassium permanganate were added and the boiling was continued for 6 or 7 minutes. The excess of permanganate was removed by adding about 15 gm. of sodium bisulfite prior to acidification and extraction. He subjected benzoic acid to the same conditions and was able to recover

98.24 and 98.17 per cent in two experiments and concluded from this that potassium permanganate did not destroy any benzoic acid. This is contrary to our findings using the more sensitive titration method.

We were reluctant about giving up the use of potassium permanganate because the subsequent chloroform extracts were always practically colorless and remained so until the definite pink end-point of titration was reached. No decidedly yellow extracts such as are rather frequent in the Folin-Flanders method were ever encountered. It was found by one of us that if a small quantity of magnesium oxide were present the effect of the permanganate in decreasing the titration value was prevented. The details of the method as we have adopted it follow:

#### *Description of the Method.*

50 cc. of urine are treated with 7.5 gm. of sodium hydroxide and 0.5 gm. of magnesium oxide in a 500 or 800 cc. Kjeldahl flask. This mixture is boiled at such a rate as to bring its volume down to approximately 25 cc. in the course of half an hour. At the end of this time, while still at the boiling temperature, 1.0 cc. of a 7 per cent solution of potassium permanganate (a solution approximately saturated at room temperature) is added, care being taken to rinse down any that may remain on the neck of the flask with the smallest possible amount of water since no unchanged permanganate must be present when the acid is subsequently added. The flask with its brown contents is twirled gently for a minute or two, cooled under the tap, a fairly closely fitting test-tube condenser placed in the neck and 30 cc. of concentrated nitric acid slowly poured in down the side of the condenser. The mixture, which rapidly clears up on the addition of the acid, is now gently boiled for 45 minutes (30 minutes are sufficient for accurate results, but a less colored, more easily titratable extract is obtained by boiling it 45 minutes) with a good current of water flowing through the condenser, cooled under the tap, and the extraction with chloroform carried out approximately according to the Folin-Flanders method. The condenser is rinsed down with 25 cc. of water to remove any benzoic acid sublimed on the bottom of the condenser, the contents of the flask are transferred to a 500 cc. separatory funnel containing 25 gm. of solid ammonium sulfate. The flask is rinsed with 20 cc. of water which is poured into the separatory funnel. After dissolving the ammonium sulfate the benzoic acid is extracted successively with one 50 cc., one 35 cc.,

and two 25 cc. portions of neutral, well washed chloroform. The first 2 portions of chloroform are used to rinse the Kjeldahl flask. The combined extracts in a second separatory funnel are washed once with 100 cc. of the Folin-Flanders salt solution (containing 1.0 cc. of concentrated HCl in 2 liters of saturated NaCl solution) and drawn off through a dry filter paper into a dry Erlenmeyer flask. The separatory funnel from which the extract was drawn is rinsed with 20 cc. of chloroform. This is drawn off into a small beaker to which the wet filter paper had been transferred. The paper is rinsed with the chloroform and the latter is poured through a dry filter into the main bulk of extract in the Erlenmeyer flask. 4 drops of 1 per cent phenolphthalein in absolute alcohol are added and the benzoic acid solution titrated to a faint, but definite pink with tenth normal sodium ethylate. The preparation and standardization of this alkali solution are adequately described in the original paper of Folin and Flanders.

We have found that the following treatment of the chloroform used in this method insures a product that is reliable as far as its neutrality is concerned:

New chloroform, which is of the U. S. P. grade and contains about 0.75 per cent of ethyl alcohol, should be washed with an equal volume of distilled water twice before being used for the extraction of benzoic acid. Chloroform which has been used in analysis and therefore contains sodium benzoate and alcohol is first filtered through a dry filter paper which removes a considerable part of the sodium benzoate in those determinations in which the titration figure was fairly large. It is now washed successively with equal volumes of tap water, once; tap water containing 5 to 10 cc. of a saturated solution of NaOH, twice; tap water, twice; and distilled water, once; six washings in all. Since the accuracy of this method depends primarily upon the use of a sample of chloroform which not only reacts neutral when tested, but which must remain neutral after being shaken with nitric acid, we have used the test which follows to determine this point:

155 cc. of chloroform, the amount used in an analysis, washed as described above, are shaken with dilute nitric acid, washed with 100 cc. of the Folin-Flanders salt solution, filtered through a dry paper, and titrated. The titration of this amount of chloroform suitable for use should not exceed 0.10 cc. of tenth normal sodium ethylate.

The application of this method or that of Folin and Flanders requires the removal of protein from the urine when this is present, as in nephritic urines. Directions for doing this have already been published, but perhaps may be repeated here.

The albuminous urine is collected in 2 per cent nitric acid which was found by Raiziss and Dubin (5) to be effective in preventing the hydrolysis of hippuric acid. 15 cc. of this dilute nitric acid are sufficient for a 3 hour nephritic urine. 50 cc. of this urine, treated with 3 or 4 drops of 0.1 per cent methyl red solution in alcohol, are brought to the first definite yellow by the addition of approximately normal NaOH. The solution is then boiled, and during the boiling sufficient one-tenth normal HCl is added to produce the first definite red color. This procedure removes the albumin nearly quantitatively so that there is no increase in the resulting titration, as has already been shown (6). The coagulum of albumin on the filter paper is washed twice with 50 cc. of boiling water. The combined washings and main bulk of filtrate are evaporated rapidly over a free flame in an 800 cc. Kjeldahl flask after being made slightly alkaline to methyl red by the addition of a small amount of dilute alkali. Bumping and frothing, should they occur, are checked by adding a glass pearl and a drop of caprylic alcohol. By supporting the funnel in the neck of the flask by means of a slice of a large cork stopper the filtration and evaporation are continued simultaneously. When the contents of the flask have been evaporated to approximately 50 cc., 7.5 gm. of NaOH and 0.5 gm. of MgO are added and the analysis made according to the directions already given.

In Table I are given the comparative results with various specimens of urine, normal and pathological, obtained by the new method and by that of Folin and Flanders. In a series of approximately half of the determinations one of us used one method and the other, the other method. The results of neither of us were known to the other until all the determinations of this series had been made, when they were compared. No. 19 in Table I is a comparison of the two methods with 50 cc. aliquots of a pure sodium hippurate solution. The only modification in this case was the reduction of the permanganate with 0.5 gm. of sodium bisulfite as a substitute for the urinary constituents which ordinarily function in this manner, prior to the acid treatment. It is noted that the agreement



between the two methods is good, as close in general as duplicates can be made by the older method, and that duplicates by the new method, where they have been made show a very close agreement.

TABLE I.

Urine No.	0.1 N Na ethylate.	
	New method.	Folin-Flanders method.
	cc.	cc.
1	4.50	4.70
	4.35	4.50
	4.80	
	4.50	
2	29.40	28.95
	29.50	29.60
3	13.95	13.55
		13.70
4	33.65	33.10
	33.50	33.80
5	1.05	0.95
6 P.*	24.55	24.80
7 P.*	26.60	26.75
8 P.*	24.50	24.50
9	14.20	14.20
10	20.10	19.80
11	8.50	8.65
12	5.25	5.35
13	8.65	8.70
14	17.55	16.95
15	8.55	8.80
16	8.95	8.80
17	12.56	12.35
18	13.75	14.00
	13.55	
19†	14.25	14.25
		13.80
		14.15

\* Urines designated by "P." are pathological specimens. All others are normal.

† 50 cc. of a sodium hippurate solution were used.

A few duplicate determinations have been made several days apart with no evidence of loss of hippuric acid in acid urines at room temperature preserved with a small amount of a 10 per cent solution of thymol in chloroform.

## CONCLUSION.

An accurate, rapid method for the determination of hippuric acid in urine is described which requires about 2 hours for completion with normal urine and about 3 hours with urine containing albumin.

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## NOTE ON A POSSIBLE SOURCE OF ERROR IN TESTING FOR BENCE-JONES PROTEIN.

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(Received for publication, June 30, 1921.)

In a long series of attempts to produce Bence-Jones proteinuria in dogs by administration of agents known to cause lesions of the bone marrow, we found a possible source of error in making qualitative tests for this protein to which it may be well to call attention. If urine, especially of dogs, containing a small amount of serum protein is allowed to stand at room temperature for from 8 to 24 hours after voiding, it will occasionally be found that the heat coagulation test, at first clear, will after standing become less marked or even disappear entirely. On the other hand a considerable cloud will still be produced by potassium ferrocyanide and acetic acid; and the addition of an equal volume of a saturated solution of ammonium sulfate will also give a precipitate. This change occurs even when the urine is preserved with toluene, and is apparently due to the proteolytic enzyme of the urine. Now since it is well known that certain digestion products give the same heat precipitation and resolution reactions as Bence-Jones protein, the desirability of using fresh urine when testing for this substance is obvious. In working with dogs it is also necessary to collect the urine by catheter, or at least to watch the dog carefully so as to be certain of obtaining urine free from contamination (vomitus, feces). Even slightly contaminated urine from the cage was several times found to contain enough digestion products to give Bence-Jones reactions, which could not be confirmed in specially collected urine.

The use of toluene as a preservative for urine may temporarily give rise to confusion. If a couple of drops of toluene are emulsified by shaking in a test-tube with urine or water an opalescent

fluid results. If this be heated it becomes clear, and when cooled the cloudiness immediately returns, and this can be repeated, thus simulating the behavior of Bence-Jones protein. Either the emulsion becomes invisible when hot on account of the change of the refractive index of the toluene, or the latter goes into solution at boiling heat and is reprecipitated upon cooling. Very little agitation, *e. g.* filtration, of urine preserved with toluene can effect sufficient emulsification to cause this phenomenon; however, by confirmatory tests it is easily differentiated.

## ACERIN.

### THE GLOBULIN OF THE MAPLE SEED (ACER SACCHARINUM).\*

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(Received for publication, June 27, 1921.)

#### INTRODUCTION.

In an earlier paper from this laboratory<sup>1</sup> it was indicated that the cotyledons of maple seed contained a high percentage of nitrogen, and preliminary experiments showed that a large amount of this nitrogen was present in the form of a globulin which could be extracted with water or with dilute solutions of sodium chloride. In the pure state the globulin is insoluble in water but owing to the presence of soluble salts in the seed it is extracted by small quantities of distilled water just as readily as by dilute solutions of sodium chloride. On diluting such aqueous extracts a large part of the globulin is precipitated.

The amount of total and soluble nitrogen in the cotyledons is shown in Table I.

The nitrogen in the 70 per cent alcoholic extract was non-protein in character, but the nature of the nitrogen compounds insoluble in dilute sodium chloride solution was not determined.

The amount of pure globulin obtained from 100 gm. of powdered seed was only about 6.5 gm. This corresponds to about only one-half of the amount of nitrogen soluble in a dilute solution of sodium chloride. However, no effort was made to secure a quantitative yield.

\* Read at the Chicago meeting of the American Association of Biological Chemists, December, 1920.

<sup>1</sup> Anderson, R. J., *J. Biol. Chem.*, 1918, xxxiv, 509.

The globulin separates on dialysis into small uniform globular particles which show no crystalline structure and it is precipitated completely from saline solutions by 0.6 saturation with ammonium sulfate. It has been isolated and purified by alternately precipitating it with ammonium sulfate and by dialyzing its dilute salt solutions. After dehydrating with alcohol and drying in vacuum it was obtained as a compact light gray or nearly white powder which was non-hygroscopic. Since this is the first protein obtained from maple seed we propose, provisionally, to call this globulin *acerin*.

TABLE I.  
*Nitrogen in Maple Seed.*

	<i>per cent</i>
Total nitrogen.....	4.40
Nitrogen soluble in 70 per cent alcohol.....	0.39
“ “ “ 5 “ “ sodium chloride.....	2.06
“ remaining in seed residue.....	1.93

TABLE II.  
*Composition of Acerin and Arachin.*

Constituents.	Acerin.	Arachin.
	<i>per cent</i>	<i>per cent</i>
C.....	51.44	52.15
H.....	6.80	6.93
N.....	18.34	18.29
S.....	0.55	0.40
O.....	22.87	22.23

A number of different preparations were made in different ways and on analysis, all gave practically identical results. These various preparations were so nearly ash-free that a non-weighable residue remained after combustion.

The percentage composition of acerin is very similar to that of legumin, amandin, or the globulin from cottonseed described by Osborne and Campbell<sup>2</sup> and Osborne and Voorhees.<sup>3</sup> However,

<sup>2</sup> Osborne, T. B., and Campbell, G. F., *J. Am. Chem. Soc.*, 1896, xviii, 609; 1898, xx, 348.

<sup>3</sup> Osborne, T. B., and Voorhees, C. G., *J. Am. Chem. Soc.*, 1894, xvi, 778.

the nitrogen distribution and particularly the percentage of basic amino-acids vary widely from results obtained by the above authors, indicating a decided difference of the molecular constitution of these proteins. Arachin, the globulin from peanut, recently described by Johns and Jones<sup>4</sup> is very similar in composition and nitrogen distribution to acerin, but the percentage of the basic amino-acids differs in the two globulins. These relations are indicated in Tables II and III.

TABLE III.  
*Nitrogen Distribution in Acerin and Arachin.*

Form of nitrogen.	Acerin.	Arachin.
	<i>per cent</i>	<i>per cent</i>
Amide nitrogen.....	2.53	2.03
Humin ".....	0.15	0.22
Basic ".....	4.86	4.96
Non-basic ".....	10.63	11.07
Basic nitrogen by Van Slyke method.		
Cystine.....	0.55	0.85
Histidine.....	1.43	1.88
Arginine.....	10.07	13.51
Lysine.....	6.07	4.98

#### EXPERIMENTAL.

The air-dried cotyledons, freed from the testa or outer brown membrane, were powdered and extracted with ether at room temperature. After the ether had evaporated, the seed residue was used for the isolation of the globulin.

A solution of the globulin is obtained on digesting this powdered material in a small quantity of water or in a dilute solution of sodium chloride. On filtering the extract through a layer of paper pulp a clear brownish yellow solution is obtained.

After extracting the powdered maple seed with 5 per cent sodium chloride solution and filtering as indicated above, the filtrate gave the following reactions: (1) It was slightly acid to litmus. (2) The globulin was precipitated on the addition of

<sup>4</sup> Johns, C. O., and Jones, D. B., *J. Biol. Chem.*, 1916-17, xxviii, 77.

dilute acids, but saturating the solution with carbon dioxide did not produce any precipitate. (3) The solution turned slightly cloudy when gradually heated to 50°C. The cloudiness increased with a rise in temperature and at 75°C. a flocculent precipitate began to form and at 100°C. the amount of the precipitate increased. (4) Ammonium sulfate added to the clear solution caused only a very faint cloudiness up to 0.2 saturation, but with 0.3 and 0.4 saturation, a heavy precipitate is produced. When this precipitate is filtered off further addition of ammonium sulfate up to complete saturation produces only a very slight cloudiness.

#### *Isolation of Acerin.*

Of the powdered ether-extracted seed, 200 gm. were digested in 900 cc. of 10 per cent sodium chloride solution to which were added 40 cc. of a saturated solution of barium hydrate. The amount of barium hydrate necessary to maintain a neutral reaction in the extract was determined by titration. The mixture was stirred for about 15 minutes and it was then filtered through a layer of paper pulp.

A perfectly clear brownish yellow filtrate was obtained. It was saturated with ammonium sulfate and the precipitate which formed was separated from the mother liquor as thoroughly as possible by centrifuging. The precipitate was dissolved by adding about 100 cc. of water and the solution was filtered through paper pulp. The globulin was again precipitated by saturating the filtrate with ammonium sulfate. The mixture was centrifuged; the precipitate dissolved in 100 cc. of water, filtered through paper pulp, and reprecipitated a third time with ammonium sulfate. After centrifuging, dissolving in water, and filtering through a layer of paper pulp, toluene was added and the solution dialyzed in a collodion bag suspended in distilled water. The water was frequently changed and the dialysis was continued until the solution was free from sulfates. The protein separated in the form of small uniform globular particles which showed no crystalline structure.

The precipitated globulin was removed from the dialyzer and collected on a Buchner funnel and washed with water. It was then suspended in 95 per cent alcohol, again filtered, and washed successively with 95 per cent alcohol, absolute alcohol, and ether,



and finally dried in vacuum over sulfuric acid. The dry substance was a heavy, nearly white powder, and it weighed 16.3 gm.

The substance was moistened with a little saturated solution of ammonium sulfate and dissolved by adding about 100 cc. of water. After filtering off a small amount of insoluble material through a layer of paper pulp the filtrate was again dialyzed. The precipitated globulin was dehydrated by treating it successively with 25, 50, 75, and 95 per cent alcohol and finally with absolute alcohol. It was filtered, washed with absolute alcohol and ether, and dried in vacuum over sulfuric acid. The dry substance weighed 13.5 gm. The substance was analyzed after drying at  $110^{\circ}$  in vacuum over phosphorus pentoxide.

0.1252 gm. substance	lost 0.0067 gm. = 5.25 per cent $H_2O$ .
0.1185 " "	gave 0.0708 " $H_2O$ and 0.2231 gm. $CO_2$ .
0.7932 " "	" 0.0351 " $BaSO_4$ .
0.1420 " "	required 18.5 cc. 0.1 N $H_2SO_4$ (Kjeldahl).

Found: C, 51.22; H, 6.68; S, 0.60; N, 18.24 per cent.

### *Second Preparation of Acerin.*

#### *Extraction with Water.*

50 gm. of the powdered, ether-extracted maple seed were digested in 200 cc. of distilled water for about 5 minutes. The extract was then filtered through paper pulp and washed with water until 200 cc. of filtrate were obtained. Ammonium sulfate was added nearly to saturation; the precipitate was centrifuged, dissolved by adding 100 cc. of water, and the solution was filtered through paper pulp and the filtrate dialyzed under toluene until free from sulfate. The precipitated globulin was dehydrated and washed with alcohol and ether as before and dried in vacuum over sulfuric acid. The yield was 3.6 gm.

There is a sufficient amount of soluble salts in the maple seed to permit practically all of the globulin to be extracted with distilled water. The seed residue in the above preparation was extracted with 200 cc. of 10 per cent sodium chloride solution, but the extract contained an exceedingly small quantity of protein.

The globulin obtained above was identical in appearance and properties with the first preparation. It was analyzed without further purification after drying at  $110^{\circ}$  in vacuum over phosphorus pentoxide.

0.1652 gm. substance lost 0.0097 gm. = 5.87 per cent  $\text{H}_2\text{O}$ .  
 0.1555 " " gave 0.0944 "  $\text{H}_2\text{O}$  and 0.2947 gm.  $\text{CO}_2$ .  
 0.8454 " " " 0.0326 "  $\text{BaSO}_4$ .  
 0.1412 " " required 18.5 cc. 0.1 N  $\text{H}_2\text{SO}_4$  (Kjeldahl).  
 Found: C, 51.68; H, 6.79; S, 0.56; N, 18.34 per cent.

### *Third Preparation of Acerin.*

200 gm. of the powdered maple seed were digested in 800 cc. of distilled water for 15 minutes, filtered, and washed with water until 800 cc. were obtained. The filtrate was saturated with ammonium sulfate and the precipitate centrifuged and then transferred to a Buchner funnel and washed with saturated ammonium sulfate solution. The precipitated globulin was redissolved by adding 250 cc. of water, the solution filtered and dialyzed until free from sulfates. The contents of the dialyzer were transferred to a beaker and allowed to settle. The supernatant liquid was poured off and the globulin brought on a Buchner funnel and washed with water. It was then suspended in about 200 cc. of water and dissolved by adding about 5 per cent of sodium chloride. The solution was filtered and dialyzed until free from chlorides. The precipitated globulin was removed from the dialyzer, dehydrated with alcohol, washed in alcohol and ether, and dried in vacuum over phosphorus pentoxide. The dry substance weighed 12.6 gm. This preparation was analyzed after drying at  $110^\circ$  in vacuum over phosphorus pentoxide.

0.1285 gm. substance lost 0.0077 gm. = 5.99 per cent  $\text{H}_2\text{O}$ .  
 0.1208 " " gave 0.0751 "  $\text{H}_2\text{O}$  and 0.2279 gm.  $\text{CO}_2$ .  
 0.7884 " " " 0.0300 "  $\text{BaSO}_4$ .  
 0.1411 " " required 18.8 cc. 0.1 N  $\text{H}_2\text{SO}_4$  (Kjeldahl).  
 Found: C, 51.45; H, 6.95; S, 0.52; N, 18.65 per cent.

### *Fourth Preparation of Acerin.*

50 gm. of the powdered maple seed were digested in 200 cc. of 70 per cent alcohol for 2 hours with frequent shaking. It was then filtered on a Buchner funnel and washed with 70 per cent alcohol until the filtrate came through colorless. The seed residue was dried in vacuum over sulfuric acid and then digested in 200 cc. of 5 per cent sodium chloride solution to which sufficient barium hydrate had been added to maintain a neutral reaction

in the extract. It was filtered through paper pulp and washed with water until 300 cc. of extract were obtained.

The extract was precipitated by adding ammonium sulfate to 0.6 saturation. The mixture was then centrifuged and the globulin transferred to a Buchner funnel and washed with ammonium sulfate solution of the same strength. The precipitate was brought into solution by adding 100 cc. of water. It was again filtered through paper pulp and dialyzed until free from sulfate. The precipitated globulin was suspended in 100 cc. of water and dissolved by adding a little ammonium sulfate. The solution was filtered and again dialyzed until free from sulfates. The globulin which separated was dehydrated with 30, 50, 70, and 95 per cent alcohol and finally with absolute alcohol and ether and dried in vacuum over phosphorus pentoxide. The nearly white powder weighed 3.3 gm.

The extraction with 70 per cent alcohol removed about 16 per cent of solid matter from the maple seed but this material contained only 0.3 per cent of non-protein nitrogen.

After precipitating the globulin from the extract obtained on digesting the seed residue in 5 per cent sodium chloride with ammonium sulfate to 0.6 saturation, further addition of ammonium sulfate to the filtered extract gave no additional precipitate, showing that all of the protein was precipitated with the above concentration of ammonium sulfate. The globulin was analyzed after drying at 105° in vacuum over phosphorus pentoxide.

0.1553 gm. substance	lost 0.0097 gm. = 6.24 per cent H <sub>2</sub> O.
0.1456 " "	gave 0.0883 " H <sub>2</sub> O and 0.2748 gm. CO <sub>2</sub> .
0.7749 " "	" 0.0314 " BaSO <sub>4</sub> .
0.1419 " "	required 18.5 cc. 0.1 N H <sub>2</sub> SO <sub>4</sub> (Kjeldahl).

Found: C, 51.46; H, 6.77; S, 0.55; N, 18.35 per cent.

#### *Fifth Preparation of Acerin.*

The seed residue, after extracting 300 gm. of maple seed with 70 per cent alcohol, was digested in 1,500 cc. of 5 per cent sodium chloride solution containing 60 cc. of Baryta water. The extract was filtered and washed with 5 per cent sodium chloride solution until about 1,600 cc. of filtrate were obtained. The clear filtrate was precipitated by adding ammonium sulfate to 0.6 saturation. After centrifuging, filtering, and washing with ammonium sulfate

solution, the globulin was dissolved by adding about 300 cc. of water. This solution was filtered and dialyzed. The globulin was removed from the dialyzer and suspended in water and dissolved by adding a small amount of ammonium sulfate. After filtering the solution it was precipitated with ammonium sulfate to 0.6 saturation, filtered, washed with ammonium sulfate solution, dissolved in about 300 cc. of water, and dialyzed until free from sulfate. After dehydrating with alcohol and ether as before it was dried in vacuum over sulfuric acid. The yield was 19.5 gm. The substance was analyzed after drying at 105° in vacuum over phosphorus pentoxide.

0.1578 gm. substance lost 0.0097 gm. = 5.89 per cent H<sub>2</sub>O.  
 0.1485 " " gave 0.0913 " H<sub>2</sub>O and 0.2801 gm. CO<sub>2</sub>.  
 0.8011 " " " 0.0309 " BaSO<sub>4</sub>.  
 0.1412 " " required 18.4 cc. 0.1 N H<sub>2</sub>SO<sub>4</sub> (Kjeldahl).  
 Found: C, 51.44; H, 6.87; S, 0.53; N, 18.24 per cent.

#### *Sixth Preparation of Acerin.*

This was prepared from 300 gm. of powdered maple seed exactly as described for the fifth preparation. The dry product weighed 20 gm. It was analyzed after drying at 105° in vacuum over phosphorus pentoxide.

0.2738 gm. substance lost 0.0160 gm. = 5.84 per cent H<sub>2</sub>O.  
 0.2578 " " gave 0.1556 " H<sub>2</sub>O and 0.4861 gm. CO<sub>2</sub>.  
 0.7552 " " " 0.0319 " Ba SO<sub>4</sub>.  
 0.1413 " " required 18.4 cc. 0.1 N H<sub>2</sub>SO<sub>4</sub> (Kjeldahl).  
 Found: C, 51.41; H, 6.72; S, 0.58; N, 18.23 per cent.

TABLE IV.

#### *Summary of Analyses of Acerin.*

Preparation.....	1	2	3	4	5	6	Average composition.
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
C.....	51.22	51.68	51.45	51.46	51.44	51.41	51.44
H.....	6.68	6.79	6.95	6.77	6.87	6.72	6.80
S.....	0.60	0.56	0.52	0.55	0.53	0.58	0.55
N.....	18.24	18.34	18.65	18.35	18.24	18.23	18.34
O (by difference)...	23.26	22.63	22.43	22.87	22.92	23.01	22.87

A composite sample containing all of the above preparations gave 0.55 per cent of sulfur and 18.32 per cent of nitrogen (Table IV), which values are identical with the above averages.

After hydrolysis according to the method of Van Slyke<sup>5</sup> the nitrogen constituents were determined as shown in Table V.

The basic amino-acids were determined in the phosphotungstic acid precipitate by the micro method of Van Slyke.<sup>6</sup> The results calculated to the basis of the original globulin are given in Table VI.

TABLE V.  
*Nitrogen Distribution in Acerin.*

Form of nitrogen.	Amount.
	<i>per cent</i>
Amide nitrogen.....	2.53
Humin " .....	0.15
Basic " .....	4.86
Non-basic " .....	10.63
Total nitrogen recovered.....	18.17

TABLE VI.  
*Basic Amino-Acids in Acerin.*

	<i>per cent</i>
Cystine.....	0.55
Arginine.....	10.07
Histidine.....	1.43
Lysine.....	6.07

#### SUMMARY.

The principal protein of the seed of the silver maple (*Acer Saccharinum*) has been isolated and purified. This protein, for which we propose the name *acerin*, is a globulin. It could not be obtained in crystalline form but separated on dialysis in small uniform globular particles. The purified acerin is a nearly white heavy powder which on combustion leaves no weighable ash. A number of different preparations were made and all of these preparations showed close agreement on analysis.

<sup>5</sup> Van Slyke, D. D., *J. Biol. Chem.*, 1912, xii, 275.

<sup>6</sup> Van Slyke, D. D., *J. Biol. Chem.*, 1915, xxiii, 407.

The average composition of acerin is as follows:

C, 51.44; H, 6.80; N, 18.34; S, 0.55; O, 22.87 per cent.

When analyzed by the Van Slyke method it was found that a considerable percentage of the basic nitrogen was present as lysine.

## DIETARY FACTORS INFLUENCING CALCIUM ASSIMILATION.

### I. THE COMPARATIVE INFLUENCE OF GREEN AND DRIED PLANT TISSUE, CABBAGE, ORANGE JUICE, AND COD LIVER OIL ON CALCIUM ASSIMILATION.\*

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(Received for publication, July 11, 1921.)

The problem of the dietary factors influencing calcium assimilation in domestic animals is not new. In 1913 we drew attention to an observation (1), which, when worked out in its details will, no doubt, have a very important bearing on the question of calcium assimilation. In this early publication data were presented which showed that there were marked differences in the amount of calcium eliminated in the feces of a milking goat when that animal was changed from old dried roughage to green pasture, and after a period of fresh green grass intake placed in the metabolism cage and returned to the dried feed ration for a calcium balance experiment. After the period of green pasture feeding, the fecal calcium elimination was so reduced as to give a positive calcium balance as compared with a high fecal calcium output and negative calcium balance on the old dry roughage. Evidently something had been ingested with the green material that allowed a more perfect skeletal storage of calcium or a more complete assimilation of this element from the intestine.

In an earlier piece of work (2) we had observed that a negative calcium balance could prevail with a lactating cow for a very long time with a slow shrinkage in milk flow but no observable physiological disturbances. This animal was under actual quanti-

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tative observation for 110 days and on a ration consisting of ordinary oat straw, wheat bran (natural or washed), rice meal, and wheat gluten. The fecal output alone of calcium was nearly equal to the daily intake of this element. Her daily output of calcium oxide was approximately 50 gm. per day with an intake of but 25 gm. The constancy in maintaining the percentage of calcium oxide in the milk was remarkable. We would not want to leave the impression that there were not deep seated and seriously abnormal conditions developing in this individual and that ultimately nutritional failure and milk production must cease, but we did not observe this in a period of 110 days. We have observed (3) disaster in reproduction with cows where probably negative calcium balances are long continued.

In this same direction Forbes and his associates (4) have made important contributions showing that high milking cows receiving rations that are supposed to provide an ample intake of calcium, nevertheless may eliminate a larger amount of calcium than is ingested. Even the addition of calcium salts to a ration of grains, dry alfalfa hay, and corn silage did not lead in their experience to the establishment of positive calcium balances.

In a similar direction Meigs, Blatherwick, and Cary (5) have contributed interesting data showing that a dry but pregnant cow is probably not assimilating sufficient calcium from a calcium-rich ration such as dry alfalfa hay, corn silage, and a grain mixture for a positive balance, but is actually transferring calcium salts from her skeleton for fetal skeleton building. Meigs and his associates are inclined to interpret these observed negative calcium balances as only temporary and merely incident to the collection of the excreta and due to nervous disturbance of the animal. While we recognize the possibility of such a factor as operative with some individuals, yet we believe that the main factor of influence in this connection is of dietary origin.

While the problem of calcium assimilation and metabolism is of very great importance in relation to growth, milk production, and egg production of our farm animals, it is of equal importance in human nutrition, and no doubt likewise related to dietary factors other than mere calcium supply. In a recent short note (6) where we briefly discussed this subject we said:



“ . . . In the case of nursing women the relation of diet to a positive or negative calcium balance and to dental conditions will assume new aspects.

“The supposition that we are dealing with something influencing calcium assimilation and which is more abundant in green than in dried plant tissue and consequently variable with the season's milk, would explain the variations in the seasonal frequency of rickets, as observed and commented upon by Hess (7).”

In continuation of this line of reasoning we are assuming that it is entirely probable that the factor or factors shown to be operative in optimum calcium assimilation in any one of our farm animals can be translated as applicable not only to other types of animals but to human nutrition as well. No doubt there will be species differences. One species, under adverse conditions will assimilate calcium more completely than another, but these differences will be quantitative and not qualitative. Just as the guinea pig is more sensitive to a lower supply of the antiscorbutic vitamine than the rat, cow, or pig, so the human infant and puppy are probably more sensitive than the rat or pig to a low supply of the dietary factors affecting calcium assimilation, but it is “a difference of degree and not of kind.”

The statement that faulty calcium assimilation or poor bone formation is due to lack of a balance of dietary factors is too indefinite to satisfy students of nutrition. Of course, it is self-evident that a low calcium- or a low phosphorus-containing ration would be a primary factor in poor skeletal development with a rapidly growing species, but the real problem before us is the disclosure of the nature of that dietary factor whose absence leads to faulty calcium assimilation even in the presence of an ample supply of this element.

Faulty calcium assimilation extending over a comparatively long time can occur in cows and mature swine without an exhibition of the quick and complete collapse shown by a growing puppy suffering from rickets, and yet we have no doubt that some of the nutritive failures exhibited by growing swine (8) and even mature swine and mature cattle on certain restricted diets will ultimately be classed as in the main a condition simulating rickets.

In this paper there will be presented only what preliminary data we have accumulated on the influence of dietary factors on calcium assimilation by the dry and milking goat. Work with other

types of animals and with food materials other than those used in these experiments is now in progress and will be reported on when the accumulated data warrant it.

#### EXPERIMENTAL.

The goats used were common American grades with no distinct breeding. They were confined in our metabolism cages with quantitative collection of the excreta. When milking animals were under observation they were milked twice daily. Analyses were applied to the weekly collection of feces and to the weekly composites of aliquots of urine and milk taken daily. Calcium determinations were made on the feeds, milk, and feces by the McCruden method, after ashing. In the urine the calcium estimation was made directly and without ashing as further suggested by McCruden (9).

#### *Record of Animal 1.*

Animal 1 was a milch goat producing 700 to 800 cc. of milk per day. She, as well as the others, was fed a grain mixture consisting of 30 parts of yellow corn, 15 of oil meal, 30 of whole oats, 24 of wheat bran, and 1 of common salt. The roughages were varied in the successive periods of observation being, respectively, alfalfa hay, oat straw, green oats, and dried green oats. In view of the fact that Forbes, Meigs, and their associates, as already stated, observed a negative calcium balance in feeding cows with alfalfa hay—a roughage distinctly high in its calcium content—we used alfalfa hay in the first period. This gave us data for comparing the behavior of the cow with the goat.

*Alfalfa Hay Period.*—After 2 weeks preliminary feeding, collections were made for a period of 5 weeks on the alfalfa ration. During this time 3,500 gm. of alfalfa and 3,500 gm. of grain mixture were consumed weekly. Contrary to expectations the animal did not go into negative calcium balance as can be seen in Table I where the record of this as well as of the other trials are tabulated. As this animal was purchased locally and had been receiving a varied and unknown ration before her purchase there is a possibility that previous storage of the factor influencing calcium assimilation had occurred which later during the alfalfa feeding period aided in maintaining normal calcium assimilation. We are in-

clined to doubt the validity of this assumption, in the first place, because the record was started in April—a time of the year when the animal kept on ordinary rations would be expected to be depleted of this dietary factor—and in the second place, because on the oat straw ration, immediately following, a negative calcium balance was readily established. It seems plausible to assume that

TABLE I.  
*Record of the Calcium Balance of Animal 1.*

Date.	Dried feces.	CaO in feces.	Feces CaO.	Urine CaO.	Milk CaO.	Intake CaO.	Output CaO.	Balance.
Alfalfa hay period.								
	gm.	per cent	gm.	gm.	gm.	gm.	gm.	
Apr. 18-25.....	2,330	2.74	63.84	0.32	12.31	86.45	76.47	+ 9.98
“ 25-May 2.....	2,240	3.01	67.42	0.18	13.72	86.45	81.32	+ 5.13
May 2-9.....	2,341	3.22	75.49	0.29	12.50	86.45	88.28	- 1.83
“ 9-16.....	2,248	3.07	69.09	0.17	15.61	86.45	84.87	+ 1.57
“ 16-23.....	2,375	2.87	68.16	0.09	11.70	86.45	79.95	+ 6.50
Oat straw period.								
June 6-13.....	1,363	1.84	25.18	0.02	10.48	12.92	35.68	-22.76
“ 13-20.....	1,222	1.70	20.74	0.10	7.39	8.69	28.23	-19.54
“ 20-27.....	1,128	1.70	19.74	0.02	8.13	8.71	27.89	-19.18
“ 27-July 4....	1,035	1.29	13.37	0.02	7.38	8.50	20.77	-12.27
Green oats period.								
July 4-11.....	888	0.68	6.06	0.02	6.20	8.04	12.28	- 4.24
“ 11-18.....	871	0.65	5.66	0.10	6.63	8.00	12.39	- 4.39
“ 18-25.....	761	1.07	8.15	0.07	6.21	7.78	14.43	- 6.65
“ 25-Aug. 1 ....	811	1.20	9.66	0.10	5.51	8.62	15.27	- 6.65
Oat hay period.								
Aug. 1-8.....	893	1.10	9.90	0.10	5.05	8.23	15.05	- 6.82
“ 8-15.....	945	1.15	10.86	0.08	4.93	8.23	15.87	- 7.64
“ 15-21.....	773	1.05	8.13	0.06	3.35	7.06	11.54	- 4.48

the alfalfa hay is considerably richer than oat straw in its content of the unknown factor influencing calcium assimilation and that the goat is less sensitive than the cow to its scarcity. In this connection it is well recognized that the goat is an animal of extraordinary persistent milking tendencies under most adverse conditions.

*Oat Straw Period.*—When feeding oat straw in place of alfalfa, casein was added to bring up the protein. As an example of a week's ration there were consumed during the second week 2,750 gm. of grain, 1,550 gm. of oat straw, and 186 gm. of casein.

During this period the calcium balance was always decidedly negative, but the decided deficit of the first week must be accepted with some reservations, as with a 6 to 8 fold decrease in calcium intake the lag of calcium excretion from the alfalfa period is decidedly contributory to the negative balance. As the pronounced negative balance persisted, there need, however, be no question as to the character of the change produced.

In the table, to bring out these facts, there are given in addition to other data both the weights of the feces and the percentage of calcium contained therein. These are important data and must be used in the interpretation of the results. A depression in the mass of feces with a rise in the percentage of calcium oxide with change of diet means nothing, but either a constancy in the fecal mass accompanied by a lowered percentage of calcium oxide or a depression in both means quite as much on a low calcium intake as does the actual establishment of a positive calcium balance. This latter will depend not only upon the kind of diet but also upon the amount of calcium in the ration.

*Green Oats Period.*—During this period, which ran for 4 weeks, fresh green oats (entire plant) were cut daily, sampled daily, and fed fresh, in amounts so that the calcium intake was approximately equal to that of the oat straw period; viz., 8 to 9 gm. On this uniform intake, though a positive balance was not established, probably because the intake was too low, the loss of calcium was reduced to approximately one-third of its former value. This has especial significance taking into consideration what was said in the preceding paragraph in regard to the importance of the relations of fecal mass to percentage of calcium contained therein, for while the fecal mass was reduced by the feeding of this succulent material, its percentage content of calcium was also reduced. Such a situation leaves no doubt as to the corrective effects brought about by the unknown factors of the fresh green roughage. Mere difference in solubility of the lime content was not the factor, as in harmony with our previous results where all the calcium in oat straw was found soluble in 0.05 N HCl, 95 per cent of

the calcium was extracted by digestion for 24 hours at 37° C. with a 0.2 per cent hydrochloric acid-pepsin solution.

*Oat Hay Period.*—During this period the green oats were substituted by the same material in the dried form as a hay, anticipating from what we expect occurs in farm practice, that this material would give about the same results as those secured with oat straw. For this purpose the oats were cut and dried in the diffused light of an attic lighted by skylights. Leaching by dew and rains was thus prevented but in addition changes induced by the fermentation of the curing process in the cock or mow were also eliminated so that the material was not strictly comparable to what is most commonly fed in general farm practice. Probably our expectations in regard to results obtained were also unwarranted, for feeding experiments with rats have shown that while oat straw contains little fat-soluble vitamine, this oat hay contained an abundance of it. So in certain relations at least, distinct differences in the effect of feeding these materials were to be expected.

3 weeks of record in the metabolism cage gave results unlike those obtained with oat straw but comparable to those secured with the green oats as seen in Table I. There was no rise in the fecal calcium elimination and no distinct difference in the calcium balance on practically the same intake. Possibly the factor influencing assimilation had not been greatly reduced in the oat hay as we dried it. Possibly our period of observation was too short. This phase will receive further study.

#### *Record of Animal 2.*

In the case of this animal our plan was to duplicate the procedure used with Animal 1. We did this in every respect but with the exception of omitting the dry alfalfa hay period. This animal was likewise milking and received the same materials as used in the first experiment. These materials consisted of the grain mixture and casein, with ordinary oat straw, green oats, or the attic dried oat hay. In all these experiments the amount of green oat hay allowed was made equivalent in dry matter to the dry matter of the oat straw. The records are shown in Table II.

These results duplicate in principle those secured with Animal 1. On the oat straw ration there was a high fecal calcium output and

a very pronounced negative calcium balance. On the green oats the dry weight of feces was practically the same as during the oat straw period but the percentage of calcium was reduced, making the total calcium elimination much less. Here a positive calcium balance was not established due to a low intake of calcium, but the degree of negative balance was greatly reduced. The effect with the oat hay was similar to our experience with Animal 1 and did

TABLE II.

*Record of the Calcium Balance of Animal 2.*

Date.	Dried feces.	CaO in feces.	Feces CaO.	Urine CaO.	Milk CaO.	Intake CaO.	Output CaO.	Balance.
Oat straw period.								
	<i>gm.</i>	<i>per cent</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	
June 17-24.....	1,547	1.50	23.26	0.03	10.86	13.34	34.15	-20.81
" 24-July 1....	1,578	1.44	22.84	0.03	8.48	13.57	31.35	-17.78
July 1-8.....	1,681	0.89	14.99	0.08	7.70	13.89	22.77	- 8.88
" 8-12.....	968	0.96	9.35	0.04	4.48	7.91	13.87	- 5.96
Green oats period.								
July 12-19.....	1,368	0.80	10.99	0.16	7.07	12.47	18.22	- 5.75
" 19-26.....	1,109	0.78	8.75	0.05	7.30	12.59	16.10	- 3.51
" 26-Aug. 2....	1,263	0.77	9.73	0.09	6.86	14.07	16.68	- 2.61
Aug. 2-8.....	1,037	0.75	7.80	0.04	5.58	11.47	13.42	- 1.95
Oat hay period.								
Aug. 8-15.....	1,509	0.59	8.90	0.05	5.75	13.83	14.70	- 0.87
" 15-21.....	1,261	0.65	8.24	0.04	4.69	11.86	12.97	- 1.11

not give the results expected; we expected an increased fecal calcium elimination and an increased negative calcium balance, but this did not result during the time of observations. Increased water intake was not a factor in these experiments since quite as much water was consumed on the oat hay ration as on the green oat ration making due allowance for its water content. For example, in a selected week on the oat hay ration the water consumption was 6,680 cc., on the green oats ration it was 5,940 cc.

*Record of Animal 3.*

Goat 3 was not a heavy producing animal, giving approximately only 200 gm. of milk per day when put on the basal ration of grain,

TABLE III.

*Record of the Calcium Balance of Animal 3.*

Date.	Dried feces.	CaO in feces.	Feces CaO.	Urine CaO.	Milk CaO.	Intake CaO.	Output CaO.	Balance.
Oat straw period.								
	gm.	per cent	gm.	gm.	gm.	gm.	gm.	
Oct. 11-18.....	1,284	1.44	18.59	3.31	0.39	16.01	22.29	-6.28
“ 18-25.....	1,736	0.92	15.97	3.24	0.26	16.01	19.47	-3.46
“ 25-Nov. 1....	1,647	0.99	16.35	3.28	0.25	16.01	19.88	-3.87
Butter fat period.								
Nov. 1-8.....	1,411	1.00	14.16	2.32	0.22	14.86	16.70	-1.84
“ 8-15.....	1,283	1.09	14.05	1.81	0.17	9.42	16.03	-6.61
Orange juice period (60 cc. per day).								
Nov. 15-22.....	1,551	0.79	12.25	2.36	0.26	12.49	14.87	-2.38
“ 22-29.....	1,291	0.75	9.74	1.74	0.29	14.32	11.77	+2.55
“ 29-Dec. 6....	1,463	0.81	11.89	1.29	0.26	14.52	13.44	+1.06
Orange juice period (120 cc. per day).								
Dec. 6-13.....	1,408	0.85	11.98	2.05	0.29	14.71	14.32	+0.39
“ 13-20.....	1,581	0.79	12.60	1.73	0.16	14.71	14.49	+0.22
Dried cabbage period (30 gm. per day).								
Dec. 20-27.....	1,467	0.81	11.92	1.63	0.24	14.64	13.79	+0.85
“ 27-Jan. 3....	1,229	0.98	12.06	2.32	0.26	16.30	14.64	+1.66
Jan. 3-10.....	1,359	0.94	12.77	1.50	0.24	15.99	14.51	+1.48
“ 10-17.....	1,371	1.01	13.94	1.65	0.22	15.99	15.81	+0.18
“ 17-24.....	1,350	0.99	12.19	2.15	0.10	10.33	14.44	-4.11
“ 24-31.....	1,119	1.11	12.43	1.39	0.12	14.56	13.94	-0.62
Feb. 1-7.....	1,221	1.18	14.42	1.44	0.19	15.75	16.05	-0.30
“ 7-14.....	1,325	1.02	13.62	2.64	0.09	15.39	16.35	-0.96
Raw cabbage period (300 gm. per day).								
Feb. 14-21.....	1,383	0.94	13.12	2.22	0.07	14.71	15.41	-0.70
“ 21-28.....	1,328	0.97	13.01	2.12	0.06	14.70	15.20	-0.50
“ 28-Mar. 7....	1,357	0.99	13.52	3.26	0.05	15.39	16.84	-1.45
Mar. 7-14.....	1,447	0.93	13.48	2.53	0.05	14.34	16.07	-1.73

TABLE III—*Continued.*

Date.	Dried feces.	CaO in feces.	Feces CaO.	Urine CaO.	Milk CaO.	Intake CaO.	Output CaO.	Balance.
Raw cabbage period (1,000 gm. per day).								
	gm.	per cent	gm.	gm.	gm.	gm.	gm.	
Mar. 14-21.....	1,019	1.12	11.47	2.19	0.08	14.01	13.74	+0.27
“ 21-28.....	829	1.47	12.23	3.36	0.08	13.65	15.67	-2.02
“ 28-Apr. 4....	928	1.30	12.12	2.77	0.07	14.87	14.96	-0.09
Apr. 4-11.....	870	1.51	13.21	2.96	0.05	13.73	16.22	-2.49
Cod liver oil period (5 cc. per day).								
Apr. 11-18.....	887	1.68	14.96	3.97	0.06	17.15	19.00	-1.85
“ 18-25.....	892	1.18	10.52	3.35	0.06	13.88	13.94	-0.06
Cod liver oil period (10 cc. per day).								
Apr. 25-May 2....	883	0.98	8.72	2.96	0.05	12.67	11.73	+0.94
May 2-9.....	670	0.95	6.39	3.38	0.05	9.83	9.52	-0.31
“ 9-16.....	Discontinued; not eating.							

casein, and oat straw. Her daily consumption was 600 gm. of grain, 40 gm. of casein, and 200 gm. of straw. On the ration, she rapidly went into negative calcium balance (see Table III).

Our results with green material, in these trials with green oats and in former work with mixed grasses, were so consistent that we believed that we were warranted at this stage of our experimentation to attempt to determine what factor in the green materials was operative in facilitating calcium retention. For this purpose our basal ration was supplemented successively with butter fat, orange juice, dried cabbage, fresh cabbage, and cod liver oil, all of which are materials which are well known to be rich in the fat-soluble vitamine or the antiscorbutic vitamine. These were selected not because we were inclined to the opinion that the factor we were dealing with was necessarily either one of these, but because we believed that this selection gave us a sufficient range of variables, including two well known factors with the possibility of the inclusion of others, so that the effect of the green oats might be duplicated. The relation of the water-soluble vitamine to this problem of calcium assimilation we felt free to disregard as our basal ration was, we believe, amply supplied with this factor.



*Butter Fat Period.*—In this period we gave 45 to 60 gm. daily of a clear filtered butter fat reducing the grain allowance by an amount equivalent in energy to the butter fat added. Butter fat feeding was continued 2 weeks. With this animal its ingestion affected the appetite adversely and milk production fell off very perceptibly, decreasing to approximately 50 cc. per day. In the short time of observation, the butter fat had no effect on the percentage of calcium eliminated by the feces, and the negative calcium balance was quite as large as on the basal ration. However, our data with butter fat are entirely too limited to give us an opinion as to whether or not it possessed any specific therapeutic value in influencing calcium assimilation.

*Orange Juice Period.*—Observations were next made with orange juice plus the basal ration. 60 cc. of orange juice were given daily for 3 weeks and then the dosage was increased to 120 cc. per day for 2 weeks. We had no difficulty in getting this animal to consume the orange juice with a complete recovery of appetite from the depressed condition experienced in the butter fat period. Milk flow was not completely restored although the daily volume now reached about 100 cc. as compared with but 50 cc. in the butter fat period. The calcium percentage in the feces was slightly but perceptibly decreased, and with the lowered milk flow the net result was a positive calcium balance. From these data alone one would be inclined to ascribe to the antiscorbutic vitamine some influence on calcium assimilation, but when, as will be shown later, no such positive calcium balances followed with the daily consumption of 1,000 gm. of fresh cabbage, also a very potent antiscorbutic material, and that with two other animals orange juice was not effective, there is left slight support for the assumption as made by Robb (10) that we were dealing with the antiscorbutic vitamine in green material as the factor influencing calcium assimilation. Possibly the orange juice we used at this time contained some of the food accessory which influences calcium assimilation.

*Dried Cabbage Period.*—Following the orange juice feeding came a long period where 30 gm. daily of dried cabbage (equivalent to about 300 gm. of fresh cabbage) were fed, plus the usual basal ration of grain mixture, casein, and oat straw. This dried cabbage was prepared by autoclaving at 15 pounds for  $1\frac{1}{2}$  hours and then

drying at 65-75° C. In this period there was a slow increase in the fecal calcium elimination, finally resulting in a negative calcium balance, although this did not take place until a lapse of 4 weeks of dried cabbage feeding. Since the behavior of this animal was indicating that the antiscorbutic vitamine might have some influence on calcium assimilation, in the next period of feeding we used fresh cabbage, feeding 300 gm. daily for 4 weeks and increasing this amount to 1,000 gm. daily for 4 more weeks with constant quantitative observations.

*Fresh Cabbage Period.*—During the period where we fed 300 gm. daily of raw fresh cabbage no reduction in the other constituents of the ration was made. The mass of feces remained practically the same as in the previous period and there was no appreciable change in the percentage of calcium in the feces. Negative calcium balance continued. When the fresh cabbage was increased to 1,000 gm. per day we reduced the straw intake from 170 to 25 gm. per day with the intention of keeping the fiber content of the ration approximately the same as in previous periods, and thereby holding the fecal mass constant. We did not succeed in this and consequently added ordinary filter paper, 60 gm. per day, for the purpose of increasing the fecal residue. With this goat the added paper did not raise the fecal residue.

The result of a lowered fecal residue during the 1,000 gm. of raw cabbage feeding was a marked increase in the percentage of calcium in the feces with the net result that just as much calcium continued to be excreted in the feces as in the previous feeding period, and a negative calcium balance continued. These results left no question as to the negative relation of the antiscorbutic vitamine to this phenomenon of calcium assimilation. While fresh cabbage or dried cabbage contains some fat-soluble vitamine (11), yet it is not particularly rich in this food factor; should it be established later that this vitamine is the one related to calcium assimilation it would be necessary to assume that cabbage was not sufficiently rich in this factor to bring about such an influence.

The failure to induce a positive calcium balance with such large amounts of cabbage is interesting when it is compared with the one positive effect of the orange juice administration. Both are rich in the antiscorbutic vitamine, and weight for weight they are

probably of approximately equal value as sources of the fat-soluble vitamine as shown by our feeding experiment with rats. Yet before attempting to make extensive analytical use of these discrepancies it is well to bear in mind that negative results must not be given too absolute a valuation as the recuperative elasticity of an animal in trials of this nature has decided limitations.

*Cod Liver Oil Period.*—The general recognition of cod liver oil as a successful therapeutic agent in rickets led us to use it in these experiments. It was emulsified with acacia gum and water and for 2 weeks this emulsion was administered in amounts equivalent to 5 cc. daily of the original oil. In the 3rd and 4th week of this period an equivalent of 10 cc. of oil was given daily. There was no influence on calcium assimilation in the 1st week, but in the 2nd week the fecal calcium oxide dropped from 1.68 to 1.18 per cent and in the 3rd week to 0.98 per cent with the output of feces practically the same as in the cabbage period. This influence of the oil resulted in establishing a positive calcium balance in the 3rd week, with a decrease in the calcium oxide of the feces from 14 to 8.7 gm. There was soon developed a distinct dislike for the oil and in the 4th week there occurred a loss of appetite with the result that the food intake was reduced in this week and a slight negative calcium balance resulted. It is safe, however, to conclude that cod liver oil was an effective agent in assisting calcium assimilation in this species.

#### *Record of Animal 4.*

*Basal Ration Period.*—While a milking goat receiving our ration of grain mixture, casein, and dry oat straw, responded readily with a negative calcium balance, we were not sure that such a reaction would follow with a dry animal. To determine this, Animal 4 was started on our basal ration October 11, 1920. She remained in positive calcium balance or equilibrium until the 2nd week in December, when a distinct negative calcium balance was assured. Feeding of this ration was continued until January 31, 1921, at which time the animal's appetite was becoming poor and her general condition apparently somewhat impaired. Her feces were hard and dry. She showed a negative calcium balance of 3.04 gm. of calcium oxide for the week. See Table IV for the record of results.



*Orange Juice Period.*—On January 31, 1921, administration of 120 cc. of orange juice daily was begun. This resulted in some stimulation to appetite and increased food consumption, but there was no change in the amount of calcium assimilated and a negative calcium balance continued. On February 14, 1921, the amount of orange juice allowed was increased to 240 cc. per day, but even this amount did not decrease the fecal calcium elimination and the animal remained at negative calcium balance.

*Cod Liver Oil Period.*—On April 4, 1921, 5 cc. of cod liver oil per day as an emulsion with acacia gum and water were given. For the first 2 weeks of oil administration there was little influence on the percentage of calcium in the feces, but with decreasing appetite and decreasing fecal output the net result was a lowered calcium elimination. This situation continued into the 3rd week with a decreasing percentage of calcium in the feces, and an actual change to a positive calcium balance in this last period. After 3 weeks on a daily allowance of 5 cc. of cod liver oil, we increased the amount to 10 cc. but the appetite of the animal had become so poor and the intake of food so low that the results of the last 2 weeks mean little. The balance experiments for this animal do show, however, that a negative calcium balance can be established with a dry goat receiving our basal ration; that orange juice had no influence on calcium assimilation; and that cod liver oil in as low amounts as 5 cc. per day did effect a better retention of the calcium of the feed.

#### *Record of Animal 5.*

*Basal Ration Period.*—The record of Animal 5 was substantially a duplication of that of No. 4, both being non-producers. The results are recorded in Table V. After prolonged feeding (2 to 3 months) a negative calcium balance was established on our basal ration. This animal as well as No. 4 was not in the metabolism cage continuously as seen in the protocols, but received the basal ration through the entire period of observation whether in the cage or not.

*Orange Juice Period.*—On February 7, 1921, after we were positive that the animal was in negative calcium balance, 120 cc. of orange juice were given daily. This was continued for 6 weeks but without any appreciable change in calcium assimilation.

The mass of feces and percentage of calcium therein remained practically like that of the preceding period.

TABLE V.  
*Record of the Calcium Balance of Animal 5.*

Date.	Dried feces.	CaO in feces.	Feces CaO.	Urine CaO.	Intake CaO.	Output CaO.	Balance.
Oat straw ration.							
	gm.	per cent	gm.	gm.	gm.	gm.	
Oct. 11-18.....	1,190	0.87	10.37	0.12	11.30	10.49	+0.81
“ 18-25.....	1,068	1.15	12.28	0.38	11.30	12.66	-1.36
“ 25-Nov. 1....	1,074	1.56	16.70	0.74	11.30	17.44	-6.14
Nov. 1-8.....	1,056	0.85	9.01	0.59	11.30	9.60	+1.70
“ 8-15.....	1,121	0.74	8.32	0.59	11.30	8.91	+2.39
Dec. 6-13.....	1,029	1.09	11.28	0.06	11.22	11.34	-0.12
“ 13-20.....	1,316	0.96	12.72	0.07	11.22	12.79	-1.57
Jan. 17-24.....	1,297	0.92	12.23	0.08	10.92	12.31	-1.39
“ 24-31.....	1,086	1.00	10.91	0.08	10.92	10.99	-0.07
“ 31-Feb. 7....	1,082	1.05	11.44	0.07	10.89	11.51	-0.62

Orange juice period (120 cc. per day).

Feb. 7-14.....	1,229	0.97	11.92	0.07	10.28	11.99	-1.71
“ 14-21.....	1,159	0.91	10.64	0.07	11.06	10.71	+0.35
“ 21-28.....	1,071	0.92	9.93	0.05	11.06	9.98	+1.08
“ 28-Mar. 7....	1,140	0.95	10.90	0.03	11.06	10.93	+0.13
Mar. 7-14.....	1,241	0.94	11.69	0.04	11.06	11.73	-0.67
“ 14-21.....	1,128	1.01	11.39	0.03	11.06	11.42	-0.36

Cod liver oil (20 cc. per day) for 2 days. Off feed.

Oat straw period.

Apr. 4-11.....	1,007	0.90	9.11	0.07	9.64	9.18	+0.46
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Cod liver oil period (5 cc. per day).

Apr. 11-18.....	894	0.84	7.52	0.06	9.64	7.58	+2.06
“ 18-25.....	846	0.75	6.42	0.06	8.65	6.48	+2.17
“ 25-May 2....	804	0.65	5.22	0.04	5.99	5.26	+0.73

*Cod Liver Oil Period.*—On March 21, 1921, we began the administration of 20 cc. of cod liver oil per day. This was continued for 2 days and so completely upset the appetite of the animal that further administration of it was discontinued. At this

point, we again reverted to the basal ration, only beginning quantitative collection of the excreta from the basal ration on April 4 to 11. This week showed a positive calcium balance which may be interpreted as the residual effect of the cod liver oil given earlier, but the decrease in fecal calcium was not marked. After reestablishing the animal on the basal ration, we continued to give 5 cc. of cod liver oil daily as an emulsion. The effect of this in reference to calcium assimilation was gradual but positive. The fecal residue decreased somewhat, while the percentage of calcium in the feces was decreased from 0.90 to 0.65 per cent, giving a distinct positive calcium balance. However, the long cod liver oil administration gradually impaired the food intake of this animal and the experiment was discontinued. The records of this animal are in accord with those of No. 4. With the basal ration a negative calcium balance was established; the added orange juice did not consistently influence calcium assimilation; the cod liver oil did decrease the calcium assimilation in the feces with the production of a distinct and continued positive calcium balance.

#### SUMMARY.

1. Experiments with goats, milking and dry, show that there is something in fresh green oats as compared with a dry oat straw which increases the amount of calcium assimilated. The oat hay, dried out of direct sunlight, but in a fairly well lighted attic, seemed to retain the properties of the fresh green oats that we were studying.

2. Orange juice administered in generous quantities (120 to 240 cc. per day) had no consistent effect on calcium assimilation.

3. Raw cabbage (1,000 gm. per day) or dried cabbage, had no influence on calcium assimilation. These data eliminate the antiscorbutic vitamine as a factor in calcium assimilation and conform with clinical experience in rickets.

4. Cod liver oil (5 to 10 cc. per day) consistently changed negative calcium balances to positive balances.

5. Our limited data show that the same factor affecting calcium assimilation and resident in green oats and grasses is present in cod liver oil.

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## A METHOD FOR THE DETERMINATION OF SUGAR IN NORMAL URINE.

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(Received for publication, June 27, 1921.)

In a previous paper<sup>1</sup> we described a procedure for the determination of sugar in normal urines based upon preliminary removal of the nitrogenous urinary constituents by means of mercury nitrate in the presence of sodium bicarbonate. The sugar was then determined in the filtrate by the use of picric acid and alkali under definite conditions. This method has been of considerable service. Frequent checkings by comparative determinations made upon the same filtrates by the Allihn gravimetric method which we have carried out have convinced us that the method is accurate for both unfermented and fermented urines. Duplicates by the colorimetric and the Allihn method practically always agree with 0.02 per cent on the sugar content of the urines.

The mercuric nitrate method has, however, a serious drawback in the laborious technique involved, which has interfered with the general usefulness of the method. We have, therefore, constantly kept in mind the development of a procedure which should permit of the determination of small quantities of sugar in urine without the use of so troublesome a technique. The method described in the present paper meets this requirement. It has been in constant use in our laboratories and elsewhere for about 2 years, and we now feel quite sure of its accuracy and reliability under widely varying conditions. While the present method retains the use of picric acid, it seems probable, as will be pointed out later, that the actual reaction takes place between the sugar and an unidentified derivative of picric acid.

<sup>1</sup> Benedict, S. R., and Osterberg, E., *J. Biol. Chem.*, 1918, xxxiv, 195.

The chief interfering substances in urine as regards the determination of sugar by picric acid (or indeed by other methods as well) are creatinine and creatine. In constructing the present method we have, therefore, had in mind primarily the elimination of interference by these compounds. The procedure developed has accomplished this end. It appears that interference by minute traces of other substances has been eliminated as well.

It has been noted in the literature that acetone causes a partial fading of the color resulting from the interaction of creatinine and picric acid in the presence of sodium hydroxide. Preliminary experiments convinced us that if the reaction between picric acid and sugar could be made to go quantitatively in the presence of hydroxide instead of carbonate, which we have had to employ heretofore, it would be possible to utilize the acetone effect on the creatinine-picric acid product, so that sugar could be determined in the presence of large amounts of creatinine or creatine. We have found that if a low concentration of picric acid is used the reaction with sugar proceeds quantitatively in the presence of sodium hydroxide. It has further been found that by addition of suitable quantities of acetone to such a mixture it is possible to destroy completely the color due to creatinine or creatine, without a serious effect on the color developed through the action of the sugar.

Before describing the exact technique of our method the following points may be mentioned as of some general interest.

The impression which quite commonly prevails that picramic acid is the colored product resulting from the interaction of creatinine and picrate in alkaline solution is incorrect. We have isolated many grams of the product of the action of picrate and creatinine, and in physical properties and stability it is quite different from picramic acid. The product of the creatinine reaction is a bright, carmine powder, which is unstable even in the dry form when exposed at all to light. When so exposed it rapidly becomes lemon yellow in color, as though reoxidized to picric acid. Analyses of the product indicate that its empirical formula is quite close to, or identical with, that of picramic acid. Nevertheless, the products are, as noted, entirely different. The procedure proposed in the present paper serves also to demonstrate the difference between the reaction products of sugar and

of creatinine with picric acid. In our process we add picric acid, alkali, and acetone to the solution, and heat. A color develops due to the acetone alone, which color fades rapidly so that in a minute or two the mixture again assumes the color of picric acid. If creatinine or creatine be present the color developed during the early stage of the heating is much intensified, so that in such a case the solution may become very intensely colored during the first minute or two of heating. Within about a minute and half, however, the color begins to fade just as does the color due to acetone alone, so that after 5 minutes of heating such solutions can scarcely be differentiated from a blank. Upon subsequent dilution these solutions have only a light yellow color as found in a blank. If sugar is present the color due to this develops more slowly than the color due to acetone or to creatinine, and does not fade or change with continued heating for at least 45 minutes. It is interesting to note that the reaction is apparently not between the sugar and the picric acid. This is indicated by the fact that during the first part of the heating in the presence of acetone, and before the sugar apparently begins to react with picric acid, it can be shown that this latter substance has been completely destroyed by the acetone. If sugar (or creatinine) be added after the heating with acetone has been carried on for a short time no color whatever develops, showing that the solution no longer contains any picric acid. If the solution after heating with the acetone until the color has faded to light yellow be acidified with hydrochloric acid and warmed, the mixture turns deep red-brown in color, and a dark precipitate forms. We have been unable to identify this product. It would seem probable that in our method the sugar reacts with an intermediate reaction product between acetone and picric acid. Creatinine will not react with this product, but reacts quickly with the original picric acid to form a compound which is not stable in the presence of acetone. It is interesting that by this adjustment of conditions it is possible to determine accurately sugar in the presence of three or more times its weight of creatinine, when under ordinary conditions creatinine yields with picric acid about three to five times as much color as does an equal weight of glucose. Indeed the reaction now proposed appears to be perhaps the most specific reduction test available for

sugar. Certainly when applied directly to urine the reaction gives more accurate results for sugar than does any other test. One may have three or four times as much creatinine or creatine present as of sugar without affecting the results. Larger amounts of creatinine may cause a slight lowering of the figure for the sugar. Hydrogen sulfide, which readily reduces pieric acid in alkaline solution under ordinary conditions may be present in relatively very large amounts (1 cc. or more of a saturated solution) without affecting the results. We had hoped that the reaction could be applied directly to urine with satisfactory results. The figures obtained in this way are, however, slightly too high, as will be evident from an inspection of Table II. We have therefore adopted a procedure of preliminary shaking of the urine with purified bone-black, which we have found removes the trace of unknown interfering substance. The bone-black used is prepared as follows. 250 gm. of commercial bone-black<sup>2</sup> are treated with about 1.5 liters of dilute hydrochloric acid (1 volume of concentrated acid diluted with 4 volumes of water) and the mixture is boiled for about 30 minutes. The bone-black is now filtered off on a large Buchner funnel and washed with water (preferably hot) until the washings are neutral to litmus. The product is then dried and powdered. The highly absorbent animal charcoals on the market should be avoided in this connection. Commercial bone-black should be used, and the final product should be tested by shaking a portion (15 cc.) of a glucose solution containing 1 mg. of the sugar in 2 cc. of water with 1 gm. of the bone-black and determining the sugar in the filtrate. There should be no detectable absorption of the sugar.

Following is the procedure for the determination of sugar in urine. The urine should be diluted so that the specific gravity does not exceed 10.25 to 10.30. 15 cc. of the urine are treated with about 1 gm. of bone-black (smaller quantities of both may be used if desired) and the mixture is shaken vigorously occasionally for a period of 5 to 10 minutes. The mixture is then filtered through a small dry filter into a dry flask or beaker. The volume

<sup>2</sup> We have employed commercial bone-black supplied by Eimer and Amend. Different samples supplied over a period of 2 years have all yielded satisfactory results. The crude bone-black must not be used without purification.

of this filtrate to be used in the determination will depend upon its sugar content, but should never exceed 3 cc. Such a volume should be used as will contain about 1 mg. of sugar. Usually 1 to 2 cc. is the right amount. The proper volume of the urine filtrate is measured into a large test-tube which is graduated at 25 cc., and if the volume used was less than 3 cc. enough water is added to make the volume exactly 3 cc. Now add exactly 1 cc. of 0.6 per cent picric acid solution (best prepared from dry picric acid) and 0.5 cc. of 5 per cent sodium hydroxide solution. Just before the tube is ready to be placed in boiling water add 5 drops of 50 per cent acetone (this should be prepared fresh every day or two by diluting some pure acetone with an equal volume of water) taking care that the drops fall into the solution and not on the sides of the tube. Shake the tube gently to mix the contents, and place immediately in boiling water and leave for 12 to 15 minutes. The standard solution should be simultaneously prepared by treating 3 cc. of pure glucose solution (containing 1 mg. of the sugar) exactly as described for the unknown solution and heating simultaneously. The pure glucose solution containing 1 mg. of the sugar in 3 cc. of solution will keep indefinitely if preserved with a little toluene. We have not been able to find a colored solution which matches the colored product of the reaction and which is permanent.

In connection with the use of the method attention may be called to the following points. The quantity of the picric acid solution used must be measured with exactness, just as are the unknown and standard sugar solutions. Slight variations in the alkali are not so important. Adding the same number of drops (about 10) to each of the tubes from the same pipette is sufficient. The acetone solution should be added last, and the tubes placed in the water bath within about a minute afterwards. The diluted acetone undergoes some peculiar change on standing which makes old solutions yield somewhat irregular results. It is therefore best to prepare the acetone solution fresh every day or two.

Each solution should be so added that it falls into the bottom of the tube, and does not hit the sides. Standard and unknown must correspond in sugar content within reasonable limits. For a 1 mg. standard satisfactory results can be obtained for an unknown solution containing between 0.75 and 1.75 mg. of sugar.

With wider variations between unknown and standard results are not so good, particularly when the quantity of sugar is low. If less than 0.7 mg. of sugar is present in the unknown it is better to have a standard solution containing 0.5 mg. of sugar in 3 cc., and to dilute both unknown and standard to 12.5 instead of to 25 cc.

TABLE I.

*Comparative Results for Sugar in Normal Urine by the Mercuric Nitrate-Picric Acid Method and by the New Procedure.*

Sample No.	Mercuric nitrate-picric acid method.		New method.	
	Before fermentation.	After fermentation.	Before fermentation.	After fermentation.
Dog urine.				
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
1	0.073	0.022	0.062	0.024
2	0.095	0.055	0.087	0.042
3	0.065	0.041	0.063	0.042
4	0.056	0.035	0.042	0.017
5	0.111	0.057	0.092	0.059
6	0.107	0.034	0.094	0.034
7	0.079	0.036	0.081	0.037
8	0.084	0.034	0.079	0.027
Human urine.				
1	0.147	0.083	0.140	0.075
2	0.077	0.033	0.069	0.033
3	0.111	0.065	0.091	0.056
4	0.086	0.043	0.077	0.043
5	0.065	0.048	0.068	0.046
6	0.064	0.034	0.068	0.046
7	0.109	0.050	0.119	0.062
8	0.211	0.068	0.220	0.060

Where the method is intelligently carried out it is very simple, and yields results of a high degree of accuracy. We have studied the procedure in detail as regards recovery of added sugar, and the determination of sugar in urine with and without creatinine and creatine addition. The results have been wholly satisfactory.

About fifty determinations have been made comparing results obtained by the new method with those given by the mercuric

nitrate procedure. A few of these results are recorded in Table I. It will be noted that the new procedure gives consistently slightly lower figures than does the old, but upon the whole the agreement is excellent between the two methods. The new method applied to the mercuric nitrate filtrates gives no lower figures than when the bone-black is employed, showing that there is no nitrogenous constituent of the urine which interferes with the method.

TABLE II.

*Comparison of the Figures Obtained for the Sugar Content of Urine with and without Preliminary Treatment with Bone-Black.*

Sample No.	Sugar.	
	With bone-black.	Without bone-black.
Dog urine.		
	<i>per cent</i>	<i>per cent</i>
1	0.078	0.100
2	0.074	0.096
3	0.072	0.096
4	0.096	0.133
5	0.110	0.144
6	0.060	0.077
7	0.119	0.168
Human urine.		
1	0.083	0.110
2	0.066	0.110
3	0.181	0.195
4	0.037	0.053

For clinical purposes the use of bone-black might be omitted if desired. Under such conditions figures will be obtained which are about 0.03 to 0.04 per cent too high. Table II shows some comparative figures with and without the use of bone-black.

We are studying the question of the adaptation of the new procedure to the determination of sugar in tissues and in blood. On account of its high degree of specificity for sugar the new procedure may prove to be of advantage in these determinations.





# CHEMICAL DEVELOPMENT OF THE OVARIES OF THE KING SALMON DURING THE SPAWNING MIGRATION.

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(Received for publication, May 24, 1921.)

Analytical data are presented here of the chemical development of the ovaries of the king salmon during the prolonged stage of its migration. Active development of the sex gonads takes place chiefly after the entrance of the salmon into fresh water. The gonad growth, therefore, occurs in the absence of an intake of food; *i.e.*, at the expense of stored materials on hand at the beginning of the migration. The migration and the time on the spawning beds preliminary to the act of spawning takes 2 to 4 months or even more. The ovaries increase in weight, during the migration of the spring run on the Columbia River, from 200 to 300 gm. at the beginning to as much as 2,500 gm. at the end.

Coincident with the growth of the ovary there is the expenditure of much dynamic energy in the migration. This dynamic energy is derived primarily from the potential energy of the excess of proteins and fats stored in the muscles in large amounts and to some extent in other organs and tissues. There is an absolute loss of mass of the muscle tissue during the migration amounting to some 45 per cent of the total. The muscle tissue left is much poorer in both proteins and fats. The fats alone drop from 20 per cent per unit mass at the beginning of the journey to less than 2 per cent at the spawning. The percentage of protein calculated on a protoplasmic basis, decreases from 20 per cent at the close of feeding to 14 per cent at the spawning. In other words, of 100 per cent of protein of muscle per unit mass of protoplasm at the beginning of migration, 30 per cent has disappeared at the spawning. Computing the loss of protein and of fat in

the tissue remaining at the spawning time and deducting that from the 45 per cent total loss during the migration, it appears that some 25 per cent or more of the muscular tissue as such has totally disappeared. These losses provide a source for a large amount of potential energy as well as for the materials that reappear in the ovaries. The foundation for these facts has been set forth in a previous publication.<sup>1</sup> They bear directly on the problem of food storage in the developing ovaries, a process that takes place coincident with the retrogressive changes in the muscle and other tissues of the salmon.

The problems undertaken in this paper are: first, the mass change in the total growth of the ovary; second, the percentage composition of the ovarian protoplasm; third, the proportionate amounts of typical stored food materials found in the eggs of the salmonoid fishes at different stages of the spawning journey; and finally, the history of the phospholipins present in such rich quantities in all eggs.

### *Historical.*

Few references have been found in the literature giving the complete analysis of the constituents of fish eggs and ovaries, and none of the ovaries of American fishes except that of Atwood's analyses of food fishes in which he gives the composition of shad roe.<sup>2</sup> Earlier preliminary reports of the work detailed in this paper represent the only data available on the chemical development of the ovaries of American fishes.<sup>3</sup> In Europe, Miescher's classic studies of the Rhine salmon<sup>4</sup> and the studies of Paton of the Scottish salmon<sup>5</sup> give data of the composition of the salmon roe as regards the store of simple and compound fats which they regard as the chief food source in the yolk.

Buttenberg<sup>6</sup> investigated the chemistry of different caviars. He also gives two analyses of fresh sturgeon roe. The averages for fresh sturgeon roe are: water 62.3, ash 1.71, nitrogenous substances (proteins) 22.8, and fat 9.9 per cent. The ash is higher than in salmon roe, the proteins are lower, and the fat content is comparable to that of the eggs of fasting salmon obtained at the spawning beds.

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<sup>1</sup> Greene, C. W., *J. Biol. Chem.*, 1919, xxxix, 435.

<sup>2</sup> Atwater, W. O., *Rep. U. S. Com. Fish and Fisheries*, 1888, 679.

<sup>3</sup> Greene, C. W., *J. Biol. Chem.*, 1918, xxxiii, p. xiii.

<sup>4</sup> Miescher, F., *Schweizerischer Fischerei-Ausstellung in Berlin*, 1880.

<sup>5</sup> Paton, D. N., *Rep. Fishery Bd. Scotland*, 1898, iv, 63.

<sup>6</sup> Buttenberg, P., *Z. Untersuch. Nahrungs- u. Genussmittel.*, 1904, vii, 233.

Rimini<sup>7</sup> also analyzed preserved fish roe, including different types of sturgeon caviar. The sodium chloride ran from 1.2 to 11 per cent, correspondingly raising the mineral content and lowering the percentages of the organic fractions. The fats of caviar ran from 14 to 28 per cent, the latter in a sample containing only 16.5 per cent of water. There is in fact no common basis of comparison between the composition of caviar as against fresh roe.

Tangl and Farkas<sup>8</sup> studied the chemical changes in the developing trout eggs. They make the following comparisons:

	Undeveloped eggs.		Developing eggs.
Water.....	66.67	66.08	65.6
Solids.....	33.33	33.92	34.94
Fat.....	0.73	7.58	7.98 Saponified fats.

These analyses indicate that the eggs lost weight, water, and energy (calories), but gained fat during incubation. The author compares the loss in trout, chicken, and spider eggs, showing the proportionate loss in each during the incubation. The trout loses the least in total weight, 5.6 per cent; the chicken egg, 17 per cent; and the spider egg, 26 per cent.

Solberg<sup>9</sup> analyzed "Dorsch"<sup>1</sup> roe (Norway), finding water 66.03 per cent, proteins 29.92 per cent, amino-acids 4.67 per cent, fat 2.26 per cent, and ash 2.16 per cent.

Kojo<sup>10</sup> gives the following analyses of the white and the yolk of the chicken's egg.

	White.	Yolk.
Water.....	87.71	49.73
Solids.....	12.29	50.27
Ash.....	0.4	1.44
Glucose.....	0.55	0.27
Nitrogen.....	1.75	2.49

The comparison between the salmon egg and the hen's egg is in the yolk. The percentage of water and total solids checks closely but the yolk of the hen's egg contains only a trifle more than half as much protein, three times as much lecithin, and somewhat more neutral fat than is contained in the salmon egg. The

<sup>7</sup>Rimini, E., *Z. Untersuch. Nahrungs- u. Genussmittel.*, 1904, vii, 232.

<sup>8</sup>Tangl, F., and Farkas, K., *Arch. ges. Physiol.*, 1904, civ, 624.

<sup>9</sup>Solberg, E., *Z. Untersuch. Nahrungs- u. Genussmittel.*, 1908, xvi, 364.

<sup>10</sup>Kojo, K., *Z. physiol. Chem.*, 1911, lxxv, 1.

important point made in Kojo's analyses is the demonstration of glucose; namely, 0.27 per cent in the yolk and 0.55 per cent in the white. Glucose is also constantly present in the salmon egg, but only to the extent of 0.09 per cent.<sup>11</sup>

#### *Collecting Stations.*

The king salmon readily available for a study of this nature are the schools which migrate up the Columbia River to spawning beds in the cold waters of the Cascades and the Rocky Mountain rivers, and those which make similar migrations in the Sacramento River in California to spawning beds in the head waters of streams arising on the slopes of Mt. Shasta. Both series have been studied but the present report is based on a collection of salmon from the Columbia River basin made in the summer of 1908.

In this series, seventeen salmon chosen from five different stations of the Columbia River and its tributaries have been analyzed in detail. The stations chosen are in order, Ilwaco at the mouth of the Columbia River; Warrendale in the Cascades, 135 miles up the Columbia; Seufert's fishery on The Dalles of the Columbia, 210 miles up; and at Ontario on the Snake River, 700 miles above the mouth of the Columbia. The spawning fish were obtained from Cazadero, some 30 miles above Portland on the San Lorenzo River. The spawners were undoubtedly of the spring migration which entered the San Lorenzo River through the Willamette River. The other samples are from spring and summer migrants.

#### *Methods of Sampling and of Analysis.*

The method of selecting salmon types and of taking and preserving samples followed in this series of fish has already been described. The analytical procedures are the same as used on muscles and are presented in a previous paper.<sup>1</sup> Samples of ovaries and of free eggs are rather difficult to pulverize and require greater precaution in extractions, especially of the lecithins. The method in brief was as follows. The sample preserved in alcohol was transferred to a Gooch crucible and extracted in a Soxhlet modified to insure extraction at the boiling point of the

<sup>11</sup> Greene, C. W., in press.

solvent.<sup>12</sup> The sample was then extracted first in alcohol, then ether, then alcohol, and finally in ether, 6 to 8 hours each. The residue was finely pulverized after the first ether extraction. The insoluble residue was dried to constant weight at 105°C. and given six extractions in distilled water. The water-soluble was evaporated and dried to constant weight, then ashed and weighed. The alcohol-ether-water-insoluble was obtained by difference and the organic extractives also by difference. The alcohol and ether were driven off the alcohol-ether-soluble fraction and the lecithins thrown down with acid chloroform-water by the method of Koch. The emulsion of lecithins and fats was oxidized, and the phosphorus determined by the official gravimetric method. The phospholipins were computed by the lecithin-phosphorus factor. Several analyses of phospholipins of critical samples were lost during the oxidations and the determinations do not reach our ideal of accuracy for the purposes. The organic extractives in the alcohol-ether-soluble fraction and from the alcohol-ether-insoluble fractions and the corresponding ashes were determined separately, but are combined in Table I. The waters were determined on a separate sample, and the neutral fats computed by difference.

#### *Growth of the Ovary.*

The growth of the ovaries in the salmon takes place chiefly during the migration. This fact is shown from the weights of the ovaries of fishes from the different stations in this series and in data obtained in the Sacramento River basin of California. The contrasts would be much more striking if sea-run fish could have been secured for the Columbia River series comparable to feeding salmon from Monterey Bay or Bolinas Bay, California.

The weights of the ovaries of fish taken in July and August from the lower Columbia River fishing stations vary from 501 to 747 gm. which compare with similar weights at tide-water on the Sacramento. The weights from the spawning beds are from 775 to 2,243 gm. for ovaries, 2,596 for ripe ova and ovaries. This represents a maximum increase of 400 to 500 per cent and is an enormous storage of food substances. Fish of the size indicative of approaching maturity, 8 to 10 kilos body weight,

<sup>12</sup>Greene, C. W., *J. Biol. Chem.*, 1909-10, vii, 503.

TABLE I.

Showing the Length, Weight, and Date of Collection, the Ovarian Weight, and the Groups of Chemical Constituents of Salmon Ovaries Collected from Five Stations, Representing Stages of the Spawning Migration, Columbia River, Season of 1908.

Collecting stations.	Date.	Fish No.	Length, tip to tip mm.	Weight.		Weight of ovaries. gm.	Sample No.	Water.		Solids.		Proteins.		Phospholipids.		Neutral fats.		Organic extractives.		Ash.			
				per cent	gm.			Total.	Fat-free.	per cent	per cent	Total.	Water-free.	Fat-free.	Sample.	per cent	per cent	Sample.	Water-free.	Fat-free.	Sample.	per cent	per cent
Ilwaco, Wash.	1908																						
	Aug. 18	1,279	870	11,215	501	1,298	54.33	63.92	45.67	36.08	25.62	56.09	30.14	3.0	6.6	3.5	15.0	32.8	1.49	3.26	1.75	0.53	
						1,300	54.33	62.66	45.67	37.34	26.03	56.98	30.02	4.1	9.0	4.7	13.3	29.1	1.64	3.59	1.89	0.57	
	"	201	281	1,035	15,445	665	1,413	53.83	62.84	46.17	37.16	25.09	54.35	29.30	4.6	10.0	5.4	14.3	31.0	46.3	17.1	71.0	
						1,414	53.83	62.33	46.17	37.67	25.75	55.77	29.81			5.3	13.6	29.5	1.59	3.45	1.84	0.55	
Warren- dale, Ore.	"	51	261	873	8,100	524	1,241	53.31	61.72	46.69	38.28	26.49	56.74	30.67	4.2	9.1	4.9	13.6	29.2	1.59	3.41	1.84	0.73
							1,242	53.31	61.66	46.69	38.34	26.37	56.47	30.50			4.9	13.5	29.0	1.69	3.62	1.95	0.85
	"	71	263	1,038	16,240	747	1,264	55.12	63.63	44.88	36.37	25.78	56.66	29.58			4.3	13.4	30.3	1.55	3.39	1.77	0.60
							1,265	55.12	63.76	44.88	36.24	25.35	56.49	29.33	3.8	8.4	4.3	13.6	30.2	1.65	3.67	1.90	0.56
Seufert's, Ore.	July 12	1,056	851	7,625	465	1,101	54.26	65.18	45.74	34.82	25.43	55.60	30.55	1.8	4.0	2.1	16.7	36.6	1.23	2.69	1.48	0.50	
							1,102	54.26	65.09	45.74	34.91	25.42	55.58	30.50			16	6.36	4.1	3.42	9.2	1.60	
	"	131	060	955	11,065	715	1,111	50.04	61.00	49.96	39.00	25.84	51.73	31.51	3.4	6.8	4.1	18.0	36.0	2.10	4.20	2.56	
							1,113	50.04	61.17	49.96	38.83	25.94	51.92	31.71			18	2.36	4.1	7.03	39.2	0.70	
	"	311	180	863	7,835	570	1,199	51.91		48.09		25.93	53.92						1.78	3.70		0.58	

Ontario, Ore.	Sept.	61, 312	940	7, 490	636	1, 496	53	35	62	04	46	65	37	96	26	29	56	36	30	57	3	9	8	5	1	6	14	0	30	0	1	45	3	10	1	68	0	93	1	99	1	08	
"	"	71, 315	1, 076	10, 410	916	1, 498	53	35	62	67	46	65	37	33	26	13	56	02	30	70	3	4	7	4	4	0	14	9	31	9	1	27	2	73	1	50	0	92	1	97	1	07	
"	"	91, 321	1, 076	14, 900	1, 899	1, 523	54	59	61	41	45	41	38	59	28	57	62	92	32	14	3	5	7	8	4	0	11	1	24	5	1	52	3	34	1	71	0	64	1	41	0	72	
"	"	111, 326	1, 034	12, 840	1, 282	1, 524	54	59	60	84	45	41	39	16	28	50	62	72	31	77	4	1	9	8	4	9	10	3	22	6	1	52	3	35	1	70	0	66	1	45	0	73	
"	"	111, 326	1, 034	12, 840	1, 282	1, 553	54	86	64	66	45	14	35	34	26	58	58	89	31	33	1	7	3	8	2	0	15	1	33	6	1	12	2	50	1	33	0	53	1	17	0	62	
"	"	111, 326	1, 034	12, 840	1, 282	1, 555	54	86	64	39	45	14	35	61	26	52	58	75	31	13	1	7	3	7	1	9	14	5	32	0	1	42	3	13	1	66	0	71	1	56	0	81	
"	"	111, 326	1, 034	12, 840	1, 282	1, 579	54	64	63	93	45	36	36	07	27	01	59	54	31	60	2	5	5	5	2	9	14	0	31	0	1	42	3	13	1	66	0	71	1	56	0	83	
"	"	111, 326	1, 034	12, 840	1, 282	1, 580	54	64	63	57	45	36	36	43	27	02	59	58	31	40	2	5	5	5	2	9	14	0	31	0	1	41	3	11	1	64	0	64	1	42	0	75	
Cazadero, Ore.	Aug.	25	1, 294	957	9, 060	1, 775	1, 429	57	24	65	23	42	76	34	77	25	89	60	55	30	00	2	3	3	1	2	2	12	2	28	6	1	57	3	67	1	75	0	76	1	77	0	86
"	"	26	1, 296	1, 054	12, 910	1, 860	1, 452	57	68	65	60	42	32	34	40	26	66	63	01	30	33	1	5	3	5	1	7	12	1	28	5	1	41	3	34	1	60	0	66	1	56	0	75
"	"	26	1, 297	986	10, 515	2, 243	1, 453	57	68	65	04	42	32	34	96	26	45	62	50	29	83	2	4	5	6	2	6	11	3	26	7	1	44	3	42	1	63	0	71	1	67	0	80
"	"	27	1, 298	887	8, 145	1, 679	1, 460	59	96	67	00	40	01	33	00	24	85	62	07	27	77	2	6	6	6	2	9	10	5	26	2	1	13	2	83	1	27	0	90	2	25	1	01
"	"	27	1, 299	887	8, 145	1, 679	1, 462	59	96	66	01	40	04	33	39	24	87	62	11	27	63	10	0	24	0	1	77	4	43	1	79	0	77	1	92	0	78	1	92	0	78		
"	"	27	1, 299	887	8, 145	1, 679	1, 473	58	19	64	02	41	91	35	98	27	06	64	73	29	78	3	6	8	6	3	9	9	1	21	7	1	37	3	27	1	51	0	65	1	55	0	77
"	"	27	1, 299	887	8, 145	1, 679	1, 474	58	19	64	08	41	91	35	92	26	95	64	46	29	68	9	2	21	9	1	46	3	49	1	61	0	59	1	61	0	59	1	41	0	65		

\*The total weight was 2,560 gm., the ripe ova free in the body cavity 1,800 gm., and the ovary with adherent ova 736 gm.

†Free ova = 1,679 gm. Ovary = 71 gm. Egg fluid = 179 gm. Total = 1,929 gm.

feeding at Monterey Bay in July, have ovaries of from 132 to 150 gm. The smallest ovaries collected at the mouth of the Columbia and at tide-water on the Sacramento River have reached 500 gm. and more in weight.

The development of the ovaries of salmon at the time of beginning the migration varies with the season. The August salmon of the mouth of the Columbia at Ilwaco are more mature as regards the sex gonads than are those from the same station that begin the migration in the spring. It is the spring run that spawn in the San Lorenzo River and apparently that migrate up the Snake River. The August fish spawn in the White Salmon River and other streams of the Cascade Mountains.

The observations seem to justify the conclusion that from 90 to 95 per cent of the total weight of the mature ovaries of the king salmon is acquired during the spawning migration; *i.e.*, while the salmon is in fresh water and not taking food. It is a unique case of synthesis and growth of the tissues of the gonads while all other organs are decreasing by a process analogous to tissue starvation.

#### *The Chemistry of the Salmon Ovaries.*

The analytical data presented in this series follows the quantitative distribution of the proteins, lipins, phospholipins, extractives, inorganic salts, and water in the ovaries and in ripe ova. The distribution of the nitrogen and of the various phosphorus fractions has been followed, but the data <sup>are</sup> ~~is~~ not now presented.

The data have been calculated on the three bases used in presenting results of the analyses of muscle. These are: first, per cent of the wet sample as collected; second, per cent of the dry or water-free sample; and third, per cent of the fat-free sample. The stored food material in the egg is out of all proportion to the active protoplasm of the germ cell or cells. This is obvious when one remembers that the protoplasm of the ovum itself is microscopic whereas the total egg is 6 to 7 mm. in diameter. Neither of the above classifications therefore can be considered as representative of the composition of the ovarian protoplasm. The data represent only the composition of stored food material, however computed.



*The Mature Ova.*

Two of the four female fish sampled at the spawning grounds contained mature ova separated from the ovarian tissue and floating free in the ovarian fluid in the body cavity. The composition of these two samples of mature eggs is as follows in terms of per cent of the total sample: water 57.68 and 58.19 per cent; total solids 42.32 and 41.91 per cent; protein 26.56 and 27.01 per cent; phospholipins 1.9 (probably low) and 3.6 per cent; neutral fats 11.70 and 9.15 per cent; total organic extractives 1.43 and 1.41 per cent; and inorganic ash 0.68 and 0.62 per cent.

This composition is characterized by its low content of salts and extractives and high content of protein. The protein is at least 70 per cent higher than in the yolk of the hen's egg. On the other hand the lecithins and neutral fats are less than half the amount stored in the yolk of the hen's egg. In the preincubation stage the organic extractives are low, much lower than in the muscle of the same fish.

*The Developing Ovaries.*

The ovaries contain ovarian tissue and developing ovules, the ovules with their stored food yolk forming an ever increasing proportion of the mass. This fact doubtless accounts for the slighter variations of composition as development progresses. The changes during development are presented under subtopics describing the various group constituents.

*The Water of the Ovaries.*

The water of the developing ovary is greatest in the mature ovary. The averages for each station are 54.08, 54.21, 52.05, 54.36, and 58.27 per cent. The increase at the spawning grounds coincides with the decreasing concentration of the blood observed in the king salmon of the Sacramento River.<sup>13</sup> It cannot be accounted for on the ground of loss of fat since the percentage of water on a fat-free basis is higher in spawning salmon.

<sup>13</sup>Greene, C. W., *Bull. U. S. Bureau Fisheries*, 1904, xxiv, 445.

*The Inorganic Salts.*

The total inorganic salts vary slightly but are a trifle higher in mature ovaries, averaging 0.57 per cent in relatively young ovaries in salmon from the mouth of the Columbia in comparison with 0.72 per cent from mature ovaries and in ripe ova. This variation is of little significance except that it gives one more confirmation of the fact that the saline content of salmon tissues is independent of the saline content of the water in which the salmon lives.

*The Proteins.*

The proteins of the developing ovaries differ little in amount from the content of ripe eggs. The station averages are: 25.62, 25.99, 25.71, 27.08, 26.10 per cent for the five stations, a variation of only 1.46 per cent. The highest protein observed was at the Ontario station and the lowest on the spawning grounds. The average for ripe eggs is 26.7 per cent which is nearly 11 per cent greater than the 16 per cent of protein present in the yolk of the hen's egg. The proteins form no inconsiderable part of the stored food of the salmon eggs.

*The Organic Extractives.*

The organic extractives of the ovaries are never great in amount. The averages are 1.55, 1.62, 1.63, 1.39, and 1.45 per cent from the five stations. The carbohydrates constitute 0.09 per cent of the extractives<sup>14</sup> and the remainder is assumed to be nitrogenous extractives. The average amount decreases slightly but not greater than the variation in duplicate determination. On the whole the ovarian extractives seem to obey the law of constant level of saturation as given for the nitrogenous extractives of muscle.<sup>15</sup>

The maintenance of the high level of protein during the growth in mass of the ovaries can only occur by the synthesis of proteins from the amino-acids of the blood and ovarian waters. The degree of saturation of the muscle waters by organic extractives amounts to 3.8 per cent, the ovarian waters are saturated only

<sup>14</sup>Greene, C. W., The glucose of salmon tissues, in press.

<sup>15</sup>Greene, C. H., *J. Biol. Chem.*, 1919, xxxix, 457.

to 2.5 per cent. While the muscle protein is being hydrolyzed protein is in process of synthesis in the developing ovaries. The loss of muscle proteins during the migration is more than enough to account for the gain in the proteins stored in the ovaries, a point supported by Paton in 1898. However, my view of the process is that the ovarian proteins are resynthesized from the hydrolytic products arising from the muscle proteins.

### *The Neutral Fats.*

The neutral fats represent the chief store of energy in the salmon's egg, just as the "egg oils" serve that function in the yolk of the hen's egg. The youngest ovaries carry the greatest amount of fats. The amounts at the three lower river fishing stations are 14.2, 13.5, and 17.4 per cent. The individual extremes are 13.3 and 18.2 per cent. Ontario salmon average 13.6 per cent with 10.3 and 15.1 per cent for the extremes. The average from the spawning grounds is 10.6 per cent. The lowest fat found was 9.1 per cent in the ripe eggs of Salmon 1,299.

The series shows a great variation in stored fats of the ovaries, just as was found for the muscles. But the spawning fish have a decidedly lower content of fat. This fact argues for the dependence of the ovarian fats on the general fat stores and on the lipolytic processes of the body of the salmon. The ovaries are not depleted of their fats to the low level of the fats of muscle, but do yield from 30 to 40 per cent of the store of fats present in the younger stages.

As with the proteins so with the egg oils, the storage takes place from the lipins liberated from supplies in the muscles, liver, connective tissues, etc. The reversible action of the lipases is adequate to account both for the storage of ovarian fats and for their percentage decrease as the fats are depleted from the body by oxidation during the migration.

### *The Phospholipins.*

The phospholipins represent the most complex food product stored in the developing ovaries. In the yolk of the hen's egg the phospholipins are present to the extent of 11 per cent and more along with about twice that amount of neutral fats. But in the

salmon ovary the amount is far less, from 2 to 3.6 per cent of the mature eggs.

The phospholipins of the ovaries from the three stations on the lower Columbia amount to 4.1, 4, and 3.4 per cent. At Ontario the average has dropped to 2.9 per cent, although one ovary contained as much as 4 per cent of phospholipins. At the spawning grounds the average phospholipin content is 2.6 per cent, with 1.9 and 3.6 per cent as the extremes.

The determinations given for the phospholipins represent the greatest variations in the data. The variations bear no direct relation to the neutral fats present in individual ovaries. However, like the neutral fats, the phospholipins decrease in amount with approaching maturity of the ova. The 4 per cent average for an ovary of 500 gm. weight drops to an average of 2.6 per cent for mature ovaries weighing 700 to 2,500 gm.

The significance of this decrease and variability is somewhat a matter of conjecture. It was argued by Paton<sup>16</sup> that the lecithins of the muscle are not adequate to provide the store found in the mature ovaries of the Scottish salmon, but loss of muscle phosphorus was equal to gain of ovarian lecithin phosphorus. The muscles of the king salmon have an average of 1.18 per cent of phospholipins when they begin the migration.<sup>1</sup> This drops to 0.44 per cent for spawning salmon. This loss, considering the large mass of the muscle tissue, is much greater than the gain to the developing ovaries. However, in view of the more recent advances in knowledge of lecithin metabolism<sup>17</sup> it is not to be assumed that the muscle lecithins are transported unchanged from muscle to ovary. It is more plausible to assume that these phospholipins are resynthesized in the ovary from available fats and phosphorus rests coming to the organs in the blood. It would be indeed difficult to prove this contention by direct tests on the salmon itself, a fact the writer keenly realizes.

On the whole it seems evident that the phospholipins play a less dominant rôle, at least they form a far less proportionate amount of the stored food materials, in the salmon ovaries and eggs than they play in the case of the hen's egg. The fats, too,

<sup>16</sup> Paton, D. N., *Rep. Fishery Bd. Scotland*, 1898, iv, 143.

<sup>17</sup> Bloor, W. R., *J. Biol. Chem.*, 1916, xxv, 577. This paper contains a full reference list.

form a smaller percentage of the food store of the egg. The dependence of the salmon on fats and fatty bodies for energy during the migration is reflected in the partial depletion of both neutral fats and phospholipins in the mature salmon eggs. The proteins are present in unusual amount and doubtless play a leading part in the nutrition of the developing embryo.



# A CHEMICAL STUDY OF CERTAIN PACIFIC COAST FISHES.\*

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(Received for publication, July 5, 1921.)

## INTRODUCTION.

In reviewing the literature on the chemical composition of fish, it is noticed that very few species have been the subject of extended investigations. The first extensive record of analyses of fish is that of Atwater published in 1888 (1). His notable investigations covered fifty-two American species, chiefly of Atlantic source but including some from the Pacific. Of his analyses, twenty-seven were based on one sample, thirteen on two samples, and twelve on from three to seven samples. Varying numbers of individuals entered into the composition of each sample. He examined nine species at different seasons of the year, the results in the case of three of these indicating seasonal variation. His figures on salmon also clearly indicate a loss in fat content during the spawning migration.

A second noteworthy investigation was conducted by Clark and Almy (2). They made a series of analyses of Atlantic Coast fishes during 1915 in order to obtain additional data on the seasonal variation both in the proximate composition and in the physical and chemical fat constants. Most of their samples were of a composite nature based on an average of three or four fish.

\* The work presented here was begun in May, 1918, under the direction of Dr. E. D. Clark and continued under Mr. A. W. Hansen until July, 1919. Since that time until June, 1920, it was carried on under the supervision of Dr. C. L. Alsberg, Chief of the Bureau. The analyses were begun at Stanford University, continued in the laboratory of the National Cannery Association at San Pedro, California, and completed at the San Diego branch of the Food Research Laboratory under the supervision of Dr. L. H. Almy.

One series was completed in the spring of 1915 and the other in the fall of the same year. They analyzed the shad before and after spawning. They also analyzed four composite samples of three fish each from the same school of weakfish. To summarize their findings briefly, they found considerable variation in many species (bluefish, butterfish, carp sucker, and weakfish) from spring to fall; the shad loses greatly in fat content during spawning; weakfish of the same school, caught at the same time, may show a wide variation in fat content (four composite samples of three fish each showed fat percentages of 1.35, 2.47, 4.88, and 8.03).

Many studies have been made of the changing composition of the king salmon during their fasting migration to the spawning grounds. In a recent publication, Greene (3) shows that spawning king salmon which have been in fresh water without eating at an estimated time of from 4 to 5 months have a fat content of 2.63 per cent and a protein content of 13.71 per cent contrasted with 16.43 per cent fat and 16.97 per cent protein in tide-water fish. Greene concludes that the king salmon stores up both fat and protein for its spawning migration.

It has frequently been pointed out that there is a relation between sea temperature and fat content of fishes. Murray and Hjort (4) stated that the fat contents of the sprat which abounds off the coast of Norway increase during summer when there is a rise in sea temperature, while both decrease toward the end of the year; it is concluded that the growth of the fish must be influenced by the prevailing temperatures in different waters.

Perhaps the most detailed investigation of the proximate composition of a selected species has been made by Johnstone (5). He selected the herring as a subject and his analyses were made of fish caught during the years 1914, 1916, and 1917. His samples were always composite ones usually based on ten fish, five males and five females. Most of the fish he analyzed were mature although he does not usually make mention of the size in connection with the analysis. However, he has made a careful record of the degree of development of the gonads in every case and hence has obtained an exact record of the relation of the chemical composition of both sexes to the sexual cycle.

In this report Johnstone remarks:



"In all races of herrings the maturation of the gonads is accompanied by an increase of fat in the flesh. For some time before the fish spawns (but after the major part of increase in the mass of the gonads has taken place) the fat contents decrease, and after spawning this decrease becomes very rapid. Between the time of spawning and the time at which maturation of the gonads begins again, the fat content of the flesh is at its minimum value."

*Procedure.*

The primary objects of our investigation were to study the seasonal variation in several species of fish and to obtain data from which food values can be ascertained. Many of the earlier analyses, based on but a few fish and made at only two or three widely separated dates, have been omitted and this report has been confined to two subjects; the variation in composition of individual fish, and the seasonal variation in the composition of the mackerel and mackerel-like fishes.

The fish used for analysis were obtained from the wholesale fish markets, from boats, or from canneries. From five to ten fish were usually selected for analysis. When the fish were small, all of the flesh was removed from one side of each, scales and bones were separated, and the remaining edible portion ground three times in a meat chopper. A sufficient quantity of the sample thus obtained was kept in a stoppered flask until the analysis was completed. In the case of larger fish, sections 1 inch or more in thickness were taken from one side of each fish and prepared for analysis as above.

Ether extract and ash were determined by the official methods.<sup>1</sup> Total solids were found by weighing out 10 gm. of the sample into a lead dish which contained a small quantity of ignited sand. After drying to constant weight in a water bath oven at 98-99°, the same sample was used for the determination of ether extract. Total nitrogen was determined by the Kjeldahl and Gunning method. The results in all cases represent the average of two closely agreeing determinations.

*Variation in the Composition of Individual Fish.*

Evidence that analyses based on but a few fish may lead to incorrect conclusions has been found in studies of several species.

<sup>1</sup> *Bull. 107* (revised), Bureau of Chemistry.

Those which have been investigated and which show marked and often erratic difference in composition are yellow fin tuna, blue fin tuna, sable-fish, barracuda, mackerel, and sardine. Data on the last named species will be presented in another paper.

In September, 1920, six yellow fin tuna (*Germo macropterus*) each weighing between 25 and 30 pounds were found to have fat percentages of 5.84, 5.71, 4.88, 2.65, 1.82, and 0.20. These six fish were selected at random from a large catch. The average fat content is 3.52 per cent although the first three fish average 5.48 per cent and the last three 1.56 per cent, a striking variation.

In September, 1918, five individual blue fin tuna showed fat percentages of 7.95, 8.71, 9.39, 10.04, and 10.76. These fish were of about the same size and were from the same boat-load. Although the variation in fat content is not striking yet the figures are valuable in showing a certain individual range in composition.

It would seem to be a natural inference that immature fish should show a lower fat content than mature fish of the same species. Some data in agreement with this inference has been found. Thus an immature sable-fish (*Anaplopoma affinis*) caught April 4, 1918, had a fat content of 0.07 per cent while a mature sable-fish, caught 2 months later, had 14.87 per cent fat. These results are in accordance with the expectation and may be characteristic of this species.

That this condition does not hold for all species was found when large, medium, and small barracuda were analyzed. Three analyses were made of this species (*Sphyræna argentea*). The first sample, consisting of ten fish caught off the coast of Lower California in December, 1918, weighing about 5 pounds apiece, had 1.85 per cent fat. The second sample, from ten fish caught at the same time and place and weighing about 2 pounds apiece, contained 6.45 per cent fat. The third, based on fish caught off San Pedro a month later, averaging 0.5 pound apiece had a fat content of 1.51 per cent.

On several occasions a number of individual mackerel from the same catch have been analyzed. These analyses, as shown in Table I, are arranged in several chronological series and the fish in each series are in an increasing order as regards weight. In the series of October 25, 1918, variation from a minimum of 0.85

per cent fat to a maximum of 7.88 per cent was found although the fish were of nearly the same size. About a month later a sample from six males had about the same composition as

TABLE I.

*Variation in the Composition of Individual Mackerel (Scomber japonicus).*

No.	No. analyzed.	Description.	Average weight.	Date.	Composition of the edible portion.				
					Solids.	Ether extract.	Ash.	Total nitrogen.	Protein (N $\times$ 6.25).
			gm.		per cent	per cent	per cent	per cent	per cent
1	1		496	Oct. 25, 1918	25.92	1.27	1.27	3.76	23.50
2	1		526	" 25, 1918	26.20	0.85	1.52	3.89	24.31
3	1		546	" 25, 1918	31.71	7.88	1.34	3.66	22.88
4	6	Males.	511	Nov. 17, 1918	24.35	0.41	1.41	3.69	23.06
5	6	Females.	511	" 17, 1918	24.89	0.85	1.37	3.74	23.37
6	1	Male spent.	582	Aug. 7, 1919	28.16	3.50	1.45	3.77	23.56
7	1	Female spent.	596	" 7, 1919	26.83	2.91	1.47	3.65	22.81
8	1	" "	596	" 7, 1919	28.03	3.87	1.47	3.69	23.06
9	1	" "	624	" 7, 1919	28.18	4.48	1.44	3.49	21.81
10	1	" full.	823	" 7, 1919	32.65	9.81	1.32	3.43	21.44
11	1	" "	1,135	" 11, 1919	30.36	6.35	1.40	3.69	23.06
12	9	Males "	1,220	" 11, 1919	30.69	7.50	1.31	3.50	21.87
13	1	Female.	538	Nov. 18, 1919	28.57	5.25	1.56	3.58	22.38
14	1	"	568	" 18, 1919	32.57	9.78	1.43	3.50	21.88
15	1	"	568	" 18, 1919	32.31	9.34	1.23	3.55	22.19
16	1	"	625	" 18, 1919	34.21	11.68	1.35	3.66	22.88
17	1	"	682	" 18, 1919	32.96	10.69	1.37	3.53	22.06
18	1	Male.	1,022	" 18, 1919	29.33	6.86	1.33	3.47	21.68
19	1	"	1,079	" 18, 1919	37.67	15.45	1.28	3.33	20.81
20	1	Female.	1,193	" 18, 1919	37.25	15.84	1.20	3.27	20.44
21	1	"	1,332	" 18, 1919	39.84	18.93	1.04	3.25	20.31
22	1	Male.	1,333	" 18, 1919	41.05	20.32	1.04	3.27	20.44
23	1		538	Dec. 10, 1919	30.65	8.41	1.23	3.52	22.00
24	1		625	" 10, 1919	33.00	10.32	1.31	3.45	21.56
25	1		681	" 10, 1919	33.99	12.35	1.35	3.45	21.56
26	1		838	" 10, 1919	37.48	17.78	1.34	3.40	21.25
27	1		938	" 10, 1919	30.31	7.77	1.52	3.45	21.56

one based on six females, both having less than 1 per cent fat. Five mackerel analyzed August 7, 1919, had fat contents increasing in much the same order as their increasing weights.

The same relation was found November 18, 1919, with the exception of Sample 18 which had a proportionately low fat content. On December 10, 1919, Nos. 23 to 26 showed close relationships between size and fat contents, while No. 27, the largest of the series, had the lowest fat content.

The data given in Table I indicate that fat content may vary widely in mackerel of the same catch and of about the same size (Nos. 1 to 3); sex appears to bear little if any relation to proximate chemical composition; although the percentage of fat generally increases with the size, Nos. 3, 18, and 27 are marked exceptions to this rule; finally, mackerel of the same size may have different fat contents at corresponding times of consecutive seasons (Nos. 1 to 5 contrasted with Nos. 13 to 17). As some of the mackerel analyzed in August, 1919, were spent (Nos. 6 to 9) and some were full (Nos. 10 to 12), it is evident that the spawning time of this species is midsummer.

#### *Seasonal Variation in the Composition of the Mackerel-Like Fishes.*

Of the various mackerel-like fishes, the species which is available throughout the year and most easily secured in the wholesale markets is the California mackerel (*Scomber japonicus*). Accordingly, most of the analyses of mackerel-like fishes have been confined to this species. As has been shown, there is considerable variation in individual mackerel and hence the samples analyzed, with the exception of a few of the earlier ones, are based on not less than five fish. It will be seen by referring to Table II that in many cases five small and five large fish of the same catch were segregated and the two composite samples analyzed separately. This was not always possible because the larger sized fish often could not be obtained. With the exception of Samples 1, 4, 5, and 6, obtained from Monterey Bay, all of the samples were taken off southern California and brought in to San Pedro. Samples 4, 5, and 6 were analyzed by Mr. A. W. Hansen of the Bureau of Chemistry.

It will be seen that of the samples averaging more than 1 kilo in weight, the two highest in fat content, Nos. 12 and 17, were in October, 1919, and February, 1920; the next highest was in August, 1919 (No. 9); while the lowest, No. 21, was in May, 1920. It has already been pointed out from the data shown in

Table I that small mackerel analyzed individually in the fall of 1918 at San Pedro were much lower in fat content than those of the succeeding fall. Samples 2 and 3 contrasted with Nos. 10 and 11 (Table II) further illustrate this fact. Considering mackerel caught during 1918-20 of from 375 to 680 gm. in weight, it is noticed that the sample of lowest fat content, No. 7, was

TABLE II.  
*Analyses of the California Mackerel (Scomber japonicus).*

No.	No. analyzed.	Description.	Average weight.	Date.	Composition of the edible portion.				
					Solids.	Ether extract.	Ash.	Total nitrogen.	Protein (N×6.25).
			gm.		per cent	per cent	per cent	per cent	per cent
1	1		400	June 3, 1918	27.17	3.62	1.27	3.50	21.87
2	3		523	Oct. 25, 1918	27.94	3.33	1.38	3.77	23.57
3	12		511	Nov. 17, 1918	24.62	0.63	1.39	3.71	23.19
4	2		505	" 1, 1918	28.61	4.64	1.46	3.58	22.37
5	4		503	" 25, 1918	25.60	1.51	1.41	3.56	22.25
6	5		348	Dec. 19, 1918	28.08	4.69	1.25	3.53	22.06
7	10	Filling.	500	May 12, 1919	23.38	0.28	1.47	3.55	22.19
8	5	1 full; 4 spent.	644	Aug. 7, 1919	28.77	4.91	1.43	3.61	22.56
9	9	All full.	1,220	" 11, 1919	30.69	7.50	1.31	3.50	21.87
10	10	Spent.	680	Oct. 9, 1919	33.83	10.16	1.35	3.68	23.00
11	5	"	450	" 20, 1919	31.69	8.49	1.37	3.61	22.56
12	5	"	1,350	" 20, 1919	38.80	18.12	1.31	3.27	20.44
13	10	Virgin.	894	Nov. 18, 1919	34.58	12.41	1.28	3.44	21.50
14	5	Filling.	724	Dec. 10, 1919	33.09	11.32	1.35	3.45	21.56
15	10	"	375	Jan. 19, 1920	27.59	4.70	1.41	3.57	22.31
16	5	"	551	Feb. 12, 1920	26.52	3.65	1.50	3.61	22.56
17	5	"	1,290	" 12, 1920	38.63	18.08	1.24	3.16	19.75
18	5	"	510	Apr. 7, 1920	27.46	4.38	1.41	3.60	22.50
19	5	"	760	" 7, 1920	30.53	7.49	1.42	3.52	22.00
20	5	"	666	May 29, 1920	25.39	1.02	1.43	3.72	23.25
21	5	"	1,370	" 29, 1920	26.97	3.45	1.38	3.59	22.44

caught in May, 1919, while No. 3, caught in November, 1918, and No. 20, caught in May, 1920 were nearly as low in percentage of fat. The two of maximum fat content are Nos. 10 and 11, caught in October, 1919. Nos. 15, 16, and 18, caught in January, February, and April, 1920, respectively, had an intermediate fat content.

We are therefore justified in concluding that the mackerel undergoes a seasonal variation in composition; that large mackerel are generally fatter than small mackerel of the same school, and that the variation in one season may not be paralleled by the next season's variation. The period of maximum fat content during 1919 followed the spawning season, while the period of minimum fat content preceded the spawning season. In the previous season the percentage of fat appeared to be at a minimum in the late fall after the spawning season.

Several analyses have been made of the other common mackerel-like fishes of southern California. With the exception of the bonita, none of the species whose analyses are shown are commonly taken in a spawning condition on this coast. It is generally considered that the blue fin tuna and albacore of the size usually taken, from 16 to 25 pounds in weight, are immature fish. For this reason the only analyses of large tuna or albacore, Nos. 4 and 5, should be considered in a class by themselves. They are quite likely mature spent fish. Such fish are not commonly taken off southern California until late in the fall. Whether such species migrate great distances, or whether they seek deep water for spawning, is as yet a mystery.

The analyses of the fish shown in Table III are too limited in number to give satisfactory information as to seasonal variation. It is apparent that considerable fat is stored in the flesh of these species during June, July, August, and September. In considering these analyses, it should be borne in mind that they are based on flesh only. The skin and heads frequently have a relatively high fat content when the amount of fat in the flesh is small. Thus, on one occasion it was found that a sample of skins, heads, and bones of albacore, even after the loss of some of their fat by cooking, had a fat content of 11.16 per cent while the per cent of fat in the flesh of the same cooked fish was 4.91. Before cooking the flesh had a fat content of 5.22 per cent (Sample 1).

#### CONCLUSIONS.

Large variations in the composition of individual fish of several species (yellow fin tuna, blue fin tuna, sable fish, barracuda, and mackerel) have been found. These variations are frequently erratic and cannot be ascribed to known factors.

TABLE III.  
*Analyses of Various Fishes of the Mackerel Family.*

No.	Species.	Average weight.	No. analyzed.	Date.	Composition of the edible portion.				
					Solids.	Ether extract.	Ash.	Total nitrogen.	Protein (N $\times$ 6.25).
		lb.			per cent	per cent	per cent	per cent	per cent
1	Albacore ( <i>Germo alalunga</i> ).....	20-30	5	June 19, 1919	32.05	5.22	1.34	4.11	25.69
2	" ".....	20-25	5	July 22, 1919	31.80	4.35	1.30	4.27	26.69
3	" ".....	20-25	5	Sept. 8, 1919	38.49	12.76	1.35	3.85	24.06
4	" ".....	40	5	Oct. 20, 1919	31.71	5.50	1.33	4.15	25.94
5	" ".....	50	5	Nov. 20, 1919	33.21	6.92	1.30	4.09	25.56
6	Blue fin tuna ( <i>Thunnus thynnus</i> ).....	23	5	Sept. 21, 1918	34.45	9.37	1.32	3.91	24.44
7	" ".....	20	5	July 22, 1919	27.43	1.08	1.43	4.02	25.13
8*	Yellow fin tuna ( <i>Germo macropterus</i> ).....	25	10	May 14, 1919	27.17	1.00	1.47	4.06	25.31
9	" ".....	30	5	Sept. 8, 1919	30.83	6.54	1.32	3.84	24.00
10	" ".....	14	3	" 15, 1920	29.27	3.29	—	4.03	25.19
11	" ".....	28	6	" 22, 1920	29.23	3.52	—	3.93	24.56
12*	" ".....	20	6	Oct. 25, 1920	26.05	0.90	—	3.89	24.31
13	Striped tuna ( <i>Gymnosarda pelamis</i> ).....	6-7	5	Aug. 18, 1919	33.48	6.62	1.28	4.08	25.50
14	" ".....	6-7	5	Oct. 23, 1919	34.64	8.11	1.32	4.05	25.31
15	Bonita ( <i>Sarda chilensis</i> ).....	9	1	Sept. 21, 1918	41.08	19.21	1.34	3.27	20.44
16	" ".....	6-7	6	May 19, 1919	26.26	1.21	1.47	3.97	24.81

\* Samples 8 and 12 were caught off Lower California. Others all from off southern California.

The variation in the composition of the mackerel during one season was not paralleled by the next season's variation.

The spawning season of the mackerel was found to be mid-summer. No evidence of a connection between decreasing fat content and the approach of the spawning season was found in the mackerel. On the contrary, the spawning season in 1919 appeared to come during a time of increasing fat content.

No evidence of a connection between the sex and the proximate chemical composition of the mackerel was found.

With some exceptions, the mackerel and the mackerel-like fishes were found to have an increasing fat content through the summer and early fall.

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## THE POTASSIUM CONTENT OF NORMAL AND SOME PATHOLOGICAL HUMAN BLOODS.\*

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(Received for publication, June 21, 1921.)

Our interest in the potassium content of human blood was aroused some time ago by the observation of Smillie<sup>1</sup> that poisoning may result from the administration of potassium salts to certain nephritic patients. This was noted in a case of nephritis and later confirmed in experimental uranum nephritis. Smillie states:

"In human beings, potassium chlorid, in doses which have no effect on normal individuals, will cause acute poisoning in individuals with chronic nephritis.

"This acute poisoning occurs because the salt, which is normally readily absorbed and very rapidly excreted, in nephritis is readily absorbed and not excreted, thus reaching a concentration in the blood which is injurious."

Owing to the fact that in the human species potassium is an important constituent of the corpuscles and present in them in a much higher concentration than in the plasma, it is quite necessary that this factor should always be taken into account in any estimation of the potassium content of human blood. The composition of the blood of different species of animals was carefully considered by Abderhalden,<sup>2</sup> and Table I is recalculated from his data. As will be noted the potassium content of whole blood in the horse, pig, and rabbit, and also man (Tables II and III) stand in marked

\* A preliminary report of these observations was made before the Society for Experimental Biology and Medicine, November 17, 1920, Myers, V. C., and Short, J. J., *Proc. Soc. Exp. Biol. and Med.*, 1920-21, xviii, 72.

<sup>1</sup> Smillie, W. G., *Arch. Int. Med.*, 1915, xvi, 330.

<sup>2</sup> Abderhalden, E., *Z. physiol. Chem.*, 1898, xxv, 106.

TABLE I.

*Potassium Content of the Blood of Different Species of Animals.\**

Species.	Potassium.	
	Whole blood.	Serum.
	mg.	per 100 cc.
Horse 1.....	227	22
“ 2.....	123	21
Pig.....	192	23
Rabbit.....	175	22
Ox.....	34	22
Bull.....	34	21
Sheep 1.....	34	22
“ 2.....	34	21
Goat.....	33	21
Cat.....	22	22
Dog 1.....	22	22
“ 2.....	21	19

\* Observations recalculated from Abderhalden.<sup>2</sup>

TABLE II.

*Potassium Content of Human Blood.\**

Case.	Diagnosis.	Age.	Sex.	Potassium.	
				Whole blood.	Serum.
				mg.	per 100 cc.
1	Normal.	25	♂	174	31
2	“	30	♀	161	33
3	Cholera.	26	♀	185	
4	“	55	♂	225	
5	“	20	♀	166	43
6	“	71	♂	203	72
7	“	23	♂	194	62
8	Diabetes.	34	♂	170	32
9	Chronic edema with albuminuria.	39	♂	116	21
10	Anasarca without albuminuria.	42	♂	190	63
11	Normal dog.			37	29

\* Observations recalculated from Schmidt.<sup>3</sup>

contrast with that found in such carnivorous animals as the cat and dog, where the findings for whole blood and serum are almost identical.

We have long possessed data on the potassium content of human blood as the result of the analyses carried out by Schmidt<sup>3</sup> in 1850. The data in Table II are recalculated from his analyses. The rather high findings for potassium in the whole blood of the cholera cases can probably be explained on the basis of the concentration of the blood found in this condition, but this would scarcely explain the high figures for the serum in two of these cases and one of the cases of nephritis. As has been pointed out by Macallum,<sup>4</sup> Schmidt's figures for the potassium content of the normal blood are likewise rather high.

Despite the fact that figures for the potassium of human blood were given by Schmidt in 1850, comparatively few data have since been recorded in the literature. A few analyses were reported by Macallum in 1917. He gives the normal potassium content of human blood plasma as 19 to 21 mg. per 100 cc., which, as will be noted, is about 60 per cent lower than the figures given by Schmidt for normal individuals. Regarding pathological cases Macallum states that his results obtained for the plasma in Bright's disease are quite incomplete but those for puerperal eclampsia are far enough advanced to furnish some points of interest. His potassium figures (four cases) are not given in absolute amount, but in relation to the sodium, taking the latter as 100. In these cases the ratio of the potassium to the sodium was increased two to four times. Since severe eclamptics generally suffer from quite pronounced acidosis, and sometimes from salt retention, it would seem logical to expect greater fluctuation in the sodium than in the potassium. On this account it is difficult to draw conclusions from changes in the ratio between the elements.

A few figures for the potassium content of blood have also been given by Drushel,<sup>5</sup> Greenwald,<sup>6</sup> Clausen,<sup>7</sup> Kramer,<sup>8</sup> and Kramer

<sup>3</sup> Schmidt, C., *Charakteristik der epidemischen Cholera gegenüber verwandten Transsudationsanomalien*, Leipsic and Mitau, 1850.

<sup>4</sup> Macallum, A. B., *Tr. College Phys. Philadelphia*, 1917, xxxix, series 3, 286.

<sup>5</sup> Drushel, W. A., *Am. J. Sc.*, 1908, xxvi, 555.

<sup>6</sup> Greenwald, I., *J. Pharmacol. and Exp. Therap.*, 1918, xi, 281; *J. Biol. Chem.*, 1919, xxxviii, 439.

<sup>7</sup> Clausen, S. W., *J. Biol. Chem.*, 1918, xxxvi, 479.

<sup>8</sup> Kramer, B., *J. Biol. Chem.*, 1920, xli, 263.

and Tisdall.<sup>9</sup> Drushel obtained 166 mg. of potassium per 100 cc. in defibrinated pig's blood, 50 mg. in sheep's blood, 20 mg. in the serum of dog's blood, and 16 mg. in dog's lymph. Greenwald found the potassium content of dog's blood (serum and whole blood) to vary from 14 to 27 mg. per 100 cc., while Clausen found the potassium content of human whole blood (pathological cases) to vary from 143 to 290 mg. per 100 cc. and in plasma from 53 to 90 mg. In his first paper, Kramer gives the normal potassium content of human serum as varying between 16 to 22 mg., while in a more recent paper Kramer and Tisdall state that the potassium content of the serum of both normal children and adults is singularly constant, the maximum variation being from 18 to 21 mg. per 100 cc. In a series of fifteen miscellaneous pathological conditions in children they report figures ranging from 23 to 70 mg. per 100 cc. of serum. The analyses reported by Kramer and Tisdall were made by a direct precipitation method without ashing. It seems rather difficult to understand why miscellaneous pathological conditions should show such a marked difference from the normal as is the case with the results of Clausen, and Kramer and Tisdall.

In 1909 Myers<sup>10</sup> carried out a study of the potassium content of the spinal fluid of insane patients. The interesting observation was made that the potassium content of spinal fluid increased very rapidly after death, as high figures being obtained one-half hour post mortem as at any later time. It was stated at that time that:

" . . . the potassium content of the cerebrospinal fluid during life corresponds very closely to the amount of potassium in the blood serum, while after death the quantity of potassium in the cerebrospinal fluid agrees more nearly with that of the whole blood."

The figures obtained for fifteen living cases varied from 14 to 28 mg. and averaged 22 mg. potassium per 100 cc. of spinal fluid. For the twenty-two specimens of spinal fluid obtained after death the findings ranging from 57 to 105 mg. with an average of 83 mg. per 100 cc.

<sup>9</sup> Kramer, B., and Tisdall, F. F., *J. Biol. Chem.*, 1921, xlyi, 339.

<sup>10</sup> Myers, V. C., *J. Biol. Chem.*, 1909, vi, 115.

The estimations of Abderhalden and Schmidt previously referred to were carried out with the chloroplatinate method, but in most recent observations, the cobalti-nitrite method has been used. Drushel, Clausen, Kramer, and Kramer and Tisdall have described adaptations of this method to blood analysis. In the last mentioned method the potassium is precipitated directly from the serum without ashing. We have used the cobalti-nitrite method of Drushel,<sup>5,11</sup> essentially as it was employed by one of us for the spinal fluid more than 12 years ago.

Our experience would lead us to believe that more satisfactory results are obtained on serum than on plasma, owing to the fact that hemolysis is more likely to take place when sodium citrate or ammonium oxalate have been added. Since human whole blood contains about ten times as much potassium as the serum it is essential to guard against the passage of any potassium from the cells. In most of our analyses the corpuscles have been separated from the serum by a double centrifuging about 2 hours after the specimens have been taken. Furthermore, when there has been any question about hemolysis, the serum has been subjected to spectroscopic examination for absorption bands of oxyhemoglobin. Slight hemolysis, however, does not necessarily greatly increase the potassium content of the serum. The potassium estimations on whole blood have been made on blood to which (potassium-free) sodium citrate was added as the anticoagulant. The analytical procedures we have employed are described below.

### *Method.*

5 cc. of blood serum or 1 cc. of whole blood are treated in a 125 cc. platinum evaporating dish with 5 cc. of a 1 to 10 sulfuric-nitric acid mixture and evaporated down rapidly in a hood over a low Bunsen burner flame. When the mixture reaches a small volume and begins to char, nitric acid is added, a few drops at a time, and the heating continued until it foams up. At this point the dish is covered with small ashless filter paper to prevent loss of material from spattering during the final steps in the oxidation. After the material has become nearly dry from the low heat, the flame is turned on full until all the organic material, including the filter paper, is burned up, and the substance is completely ashed.

The ash is now dissolved in 3 to 5 cc. of hot water, 1 to 2 cc. of glacial acetic acid added, and then transferred to a 50 cc. beaker with the aid of

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<sup>11</sup> Drushel, W. A., *Am. J. Sc.*, 1907, xxiv, 433.

TABLE III.  
*The Potassium Content of Normal and Some Pathological Human Bloods.*

Case	Age	Sex	Potassium.		Total solids.	Red cells.	Chlorides as NaCl.	Other blood analyses.					Clinical diagnosis.
			Serum	Whole blood.				Uric acid.	Urea N.	Creatinine.	Sugar.	CO <sub>2</sub>	
			mg. per 100 cc.	mg. per 100 cc.	per cent.	millions	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	per cent.	cc.	
1. F. W.	32	♂	14	158	19.3								Normal.
2. G. B.	26	♂	18	269	21.6								"
3. L. S.	20	♂	18	195	21.4								"
4. C. W.	26	♂	18	141									"
5. M. D.	26	♂	10	52	13.4		537	5.1	43	5.5	0.121	52	Chronic diffuse nephritis, died.
6. M. B.	41	♀	15	134	14.7	3.1	531	7.0	102	8.3	0.159	33	Chronic interstitial nephritis.
7. S. T.	39	♀	17	88	13.3	3.6	594	9.3	100	12.0	0.136	20	" diffuse nephritis, uremia.
8. T. D.	50	♀	18		13.3	3.1		6.5	95	5.7	0.142	29	Chronic interstitial nephritis.
9. M. W.	30	♂	19	94	11.7	4.0	444	9.7	165	13.1	0.160	26	"
10. J. S.	17	♂	16	96	15.6	4.0	507		53*	3.6	0.150	51	died.
													Advanced nephritis, toxico-
													sis, improved.
11. B. G.	27	♂	20	94	14.2	2.2	435		115	16.0	0.163		Chronic nephritis, convulsions, died.
12. C. C.	32	♀	28*	59	12.0	1.7	469	18.4	214	25.0	0.328	52	Chronic diffuse nephritis, died.
13. A. B.	32	♀	28†	148	18.7	4.1	531	4.3	21	3.0	0.121		Chronic interstitial nephritis, hyperthyroidism.

14. F. M.	57	♂	35†	81	15.7	2.1	568		59	4.8	0.153	Double polycystic kidney, secondary anemia.
15. H. K.	30	♂	20	146			519		12	2.2	0.145	Cardiovascular disease.
16. C. O'N.	52	♀	20				494		10			Cardiac decompensation, ana- sarcia.
17. J. S.	41	♂	15			4.5	488	3.1	19		0.143	Essential hypertension.
18. S. D.	50	♀	28†	168	21.0		475		11	2.2	0.101	"
19. M. C.	47	♂	16			4.9	450	5.1	25	2.2	0.145	Carcinoma of esophagus.
20. J. S.	47	♂	16				488		18		0.143	Lobar pneumonia, conva- lescent.
21. R. W.	24	♀	17				550		7	2.5	0.130	Eclampsia.
22. F. W.	55	♀	24†						18	2.3	0.510	Diabetic coma, died.
23. A. B.	50	♀		124	19.3	3.5						Pernicious anemia.
24. M. T.	46	♀	18	72		1.8						Syphilis, pernicious anemia (?).

\* Blood taken from heart about 45 minutes after death.

† Specimens taken before the necessity for the almost immediate separation of serum from the clot was fully appreciated.

a rubber-tipped rod. 10 cc. of the sodium cobalti-nitrite reagent are added, the beaker is covered with a watch-glass and placed in the refrigerator at 4°C. over night. On the following day the contents of the beaker are filtered through a porcelain Gooch crucible with thick asbestos mat, and washed with about 100 cc. of ice cold water.

The asbestos mat is now removed with a stirring rod and placed in a beaker containing 10 cc. of 0.1 N potassium permanganate and about 100 cc. of distilled water nearly at the boiling point and stirred. The crucible is then immersed in the hot permanganate solution in order to oxidize the last trace of the precipitate which may have adhered to it. The solution is now heated for 5 or 6 minutes until manganese hydroxide begins to separate out and the solution darkens. 10 to 15 cc. of 1 to 7 sulfuric acid are next added, and the solution, after stirring, is allowed to stand for several minutes. A known excess of 0.1 N oxalic acid (generally 10 cc.), containing 50 cc. of strong sulfuric acid to the liter, is then run into the beaker. After the permanganate color is thoroughly bleached, the crucible is removed, washing it with hot water. The hot solution is now titrated to color with the permanganate, the excess of permanganate over the oxalic representing the amount of potassium. After the titration the asbestos is again transferred to the Gooch crucible, washed with hot water, and the crucible set aside for the next determination.

Drushel has calculated that 1 cc. of 0.1 N potassium permanganate is required to oxidize 0.707 mg. of potassium in the form of  $K_2NaCo(NO_2)_6 \cdot H_2O$ . We have been able to use this factor in a large number of potassium estimations we have made on urine, feces, and muscle, but where one is working with very small amounts of potassium, as in the case of blood, it has generally been found desirable to employ an empirical factor. This was done by Adie and Wood<sup>12</sup> and by Myers<sup>10</sup> for spinal fluid. Such a factor has been worked out with each cobalti-nitrite solution for the amount of potassium determined (generally 1 mg.) under the conditions employed. Analyses have been made in duplicate.

Our observations are given in Table III.

#### DISCUSSION.

No evidence of a retention of potassium was found in the serum or whole blood of the seven cases of advanced nephritis given in Table III; all of these cases showed marked nitrogen retention. This does not exclude the possibility, however, that potassium retention may occur in cases with edema and marked chloride retention but without nitrogen retention. It may be noted that in Case 7, which showed considerable chloride as well as

<sup>12</sup> Adie, R. H., and Wood, T. B., *J. Chem. Soc.*, 1900, lxxvii, 1076.



nitrogen retention, the serum contained a normal amount of potassium.

According to a few original observations reported and others cited by Blumenfeldt<sup>13</sup> it appears that in certain cardiac and renal conditions there may be a retention of potassium with an increase of this element in the tissues. In view of our findings, and considering the fact that potassium is chiefly a constituent of body tissues rather than of body fluids, a comparison of the potassium content of the corpuscles, or of the whole blood on the basis of its hemoglobin content, would be more likely to disclose pathological variations than the method employed. It may be noted here that observations on the potassium content of the muscle tissue of the rabbit<sup>14</sup> have shown that starvation, or diets low in potassium, may reduce the potassium concentration of the muscle as much as 40 per cent.

As will be noted, none of our pathological cases appear to disclose any increase in the potassium content of the serum. In this respect our findings differ from the reported figures of Clausen, and Kramer and Tisdall.

It has recently been pointed out by Van Slyke and Cullen,<sup>15</sup> and Fridericia<sup>16</sup> that a shifting of the chlorides and CO<sub>2</sub> takes place when plasma is allowed to stand in contact with the cells, the chlorides of the plasma generally being increased. Since chloride estimations in the plasma are of value only when the plasma is separated as soon as the blood is drawn, Myers and Short<sup>17</sup> have recommended the estimation of the chlorides on whole blood. If immediate separation of the plasma from the corpuscles is necessary to prevent a change (increase) in the plasma chlorides, even though the chloride content of the cells is below that of the plasma, it is logical to believe that a rapid separation of the serum (or plasma) might be even more necessary in the case of the potassium, owing to the fact that the cells contain roughly twenty times as much potassium as the plasma. It is evident from the observations previously reported on spinal fluid that the permeability of certain cell membranes for potassium may change very rapidly after death.

<sup>13</sup> Blumenfeldt, E., *Z. exp. Path. u. Therap.*, 1913, xii, 523.

<sup>14</sup> Myers, V. C., unpublished observations.

<sup>15</sup> Van Slyke, D. D., and Cullen, G. E., *J. Biol. Chem.*, 1917, xxx, 317.

<sup>16</sup> Fridericia, L. S., *J. Biol. Chem.*, 1920, xlii, 245.

<sup>17</sup> Myers, V. C., and Short, J. J., *J. Biol. Chem.*, 1920, xlii, 47.

## CONCLUSIONS.

The potassium content of normal human blood serum amounts to rather less than 20 mg. per 100 cc., while for whole blood the figures are eight to twelve times this amount.

Owing to the high content of potassium in the cells, precautions should be taken in the analysis of serum or plasma to make the separation before any transfer of the potassium from the cells has taken place. We believe that the serum is preferable to the plasma for this determination. The separation of the serum should be made as soon as possible after the blood is withdrawn.

The potassium content of whole blood is roughly proportional to the total solid and red cell content.

In our series of seven nephritics with marked nitrogen retention no increase in the potassium content of the serum or whole blood was noted. On the contrary, the potassium content of the whole blood was diminished, apparently due in large part to an associated secondary anemia. These few observations do not lend support to the suggestion of Smillie that some of the symptoms of uremia may be due to a potassium poisoning as a result of retention of this element.

In none of our pathological cases were abnormal figures for the potassium of the serum found when the serum was separated within 2 hours after the blood was drawn.

# A CHEMICAL STUDY OF THE CALIFORNIA SARDINE (*SARDINIA CÆRULEA*).

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(Received for publication, July 5, 1921.)

## INTRODUCTION.

The limited value of two or three analyses of each of a large number of species of fish has been emphasized in a previous paper. While making the study of Pacific Coast fishes it became apparent that it would be impossible to secure extensive information regarding the composition of more than a few species in the time allowed, and accordingly there was suggested the advisability of securing a large amount of data on a single species. The sardine was chosen because of its commercial importance, its availability, and the ease of sampling due to its small size. The methods of preparing and analyzing samples were the same as those described in the preceding paper.

At San Pedro, although sardines are packed every month in the year, most of the pack is put up from December to April. Sardines of commercial importance vary in weight from 15 to 250 gm. and are generally roughly divided into four groups. The smallest sardines, spoken of as "quarters" are packed in rectangular cans of about  $\frac{1}{4}$  pound capacity. Sardines of the next larger size, "halves," are generally packed in cans of  $\frac{1}{2}$  pound capacity. The third size is packed in oval cans of nearly 1 pound capacity which hold from seven to ten fish of this group. These sardines are called "small ovals." The fourth and largest size, "large ovals," is packed in the same can as the third but only four or five fish are required to fill the can. If one bears in mind that nearly one-half of the fish is removed during preparation for packing, a fair idea of the size of the fish in each group can be had.

The relative abundance of the different sizes varies in different seasons and in different localities. As a rule, "ovals" appear during December off southern California and disappear in June. During the first 6 months of 1919, "quarters" and "halves" were fairly abundant at San Pedro while few were to be had at San Diego. During the corresponding period of 1920, the reverse was the case; in fact it became necessary to discontinue the analyses of the small sardines because of the lack of samples.

As regards maturity, few if any of the smallest size are mature, while some of the second size, most of the third size, and all of the fourth size are mature. It will be seen then that a sardine which is mature so far as reproductive ability is concerned is not necessarily full grown.

#### *Results of Analyses.*

Unless otherwise stated, all of the sardine analyses have been based on composite samples of ten fish each. As will be noted in Tables I and II there is a wide variation in the composition of sardines depending in part on the size, maturity, and season. In order to find what individual differences might be expected at a time when the fat content is low, ten individuals of about the same size were analyzed separately on April 21, 1919. The results are given in Table III.

Since sardines of the two smaller sizes are generally immature, it appeared that the segregation of data on these two sizes might give information on the variation in composition as related to factors other than those connected with spawning. The monthly averages of the analyses of sardines in these two groups are shown in Table I. In Tables II and IV are shown similar analyses of "small ovals" and "large ovals" respectively.

Beginning in January, 1920, from six to nine samples of ten fish each were obtained about every 10 days. All of the fish of a given sample were of approximately the same weight and the average weight of the samples ranged from 80 or 100 gm. to 220 or 240 gm. in 20 gm. intervals. During this period, in preparing the samples for analysis, the gonads were carefully segregated and their degree of development ascertained by finding the ratio of their weight to the weight of the whole fish from which they were derived. The analyses which were made during the transition period from high

to low fat content are shown in Table V. The analyses of March 29, shown in this table, were from a school of fish which were still rather fat, while 10 days later and also 14 days later fish of quite different composition were found.

TABLE I.  
*Monthly Average Composition of Small Sardines.*

Month.	Average weight per fish.	No. of analyses.	Composition of the edible portion.			
			Solids.	Ether extract.	Total nitrogen.	Protein (N $\times$ 6.25).
"Quarters." Average weight 15 to 40 gm.						
<i>1918</i>	<i>gm.</i>		<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Oct.....	30.4	1	22.80	0.48	3.31	20.69
Nov.....						
Dec.....	20.1	2	23.64	1.58	3.26	20.37
<i>1919</i>						
Jan.....	34.1	2	21.25	0.70	3.15	19.68
Feb.....	24.3	4	20.78	0.52	3.07	19.19
Mar.....						
Apr.....	32.1	2	22.98	1.06	3.34	20.88
May.....	28.2	2	23.57	1.47	3.32	20.75
June.....						
July.....	35.9	2	25.85	3.34	3.37	21.06
Aug.....	29.8	1	27.18	3.36	3.51	21.94
Sept.....	24.0	1	25.52	3.39	3.38	21.12
"Halves." Average weight 48 to 70 gm.						
<i>1918</i>						
Dec.....	54.6	2	26.05	3.05	3.34	20.88
<i>1919</i>						
Jan.....	61.8	2	23.67	3.11	3.13	19.56
Feb.....	51.0	1	16.88	0.34	2.41	15.06
Mar.....	48.1	1	23.44	2.66	3.06	19.12
Apr.....	60.5	2	23.42	3.21	3.21	20.06
May.....	69.0	1	20.21	0.39	3.04	19.00
June.....						
July.....	59.6	2	27.85	5.64	3.37	21.06
Aug.....	62.3	1	28.92	6.87	3.42	21.37

In studying the possible influence of development of gonads on the proximate composition of the flesh, several analyses of ovaries and testes were made; a record of the development of the gonads compared to the body weight was kept; and on two

TABLE II.  
*Monthly Average Composition of "Small Ovals."*

Month.	Average weight per fish.	No. of analyses.	Composition of the edible portion.			
			Solids.	Ether extract.	Total nitrogen.	Protein (N $\times$ 6.25).
Average weight 80 to 120 gm.						
1918	gm.		per cent	per cent	per cent	per cent
Dec.....	105.3	3	26.93	5.38	3.18	19.87
1919						
Jan.....	103.2	4	30.25	9.62	3.10	19.37
Feb.....	85.8	2	23.27	2.95	3.08	19.25
Mar.....	99.1	1	28.88	7.44	3.20	20.00
Apr.....	85.5	2	20.65	0.77	3.11	19.09
May.....	93.7	2	21.31	0.60	3.11	19.09
Dec.....	86.5	2	32.25	12.06	3.03	18.93
1920						
Jan.....	100.0	3	31.68	10.74	3.17	19.81
Feb.....	104.0	5	32.12	11.66	3.09	19.31
Mar.....	104.0	10	30.17	9.49	3.12	19.15
Apr.....	105.0	8	26.20	4.50	3.21	20.06
May.....	113.0	3	26.20	3.69	3.40	21.25
June*.....	100.0	3	27.67	5.07	3.45	21.56

\* All June analyses were made June 3, as no sardines of this size could be obtained later in the month.

TABLE III.  
*Variation in the Composition of Individual Sardines.*

No.	Sex.	Average weight.	Composition of the edible portion.				
			Solids.	Ether extract.	Ash.	Total nitrogen.	Protein (N $\times$ 6.25).
		gm.	per cent	per cent	per cent	per cent	per cent
1	Male.	162	20.92	0.52	1.59	3.03	18.94
2	"	170	20.89	0.24	1.63	3.09	19.31
3	"	165	19.07	0.13	1.80	2.87	17.94
4	Female.	184	25.92	4.66	1.46	3.23	20.19
5	"	172	21.43	0.39	1.68	3.07	19.19
6	"	162	22.09	0.80	1.47	3.11	19.44
7	"	160	21.71	0.09	1.59	3.21	20.06
8	"	162	24.14	1.80	1.57	3.36	21.00
9	"	152	20.12	0.10	1.66	3.07	19.19
10	"	162	20.31	0.13	1.57	2.99	18.69
Average .....		165	21.66	0.89	1.60	3.10	19.37

occasions ten relatively mature and ten relatively immature fish of each sex were segregated and analyzed. These results are shown in Tables VI, VII, and VIII, respectively.

TABLE IV.  
*Monthly Average Composition of "Large Ovals."*

Month.	Average weight per fish.	No. of analyses.	Composition of the edible portion.			
			Solids.	Ether extract.	Total nitrogen.	Protein (N× 6.25)
Average weight 140 to 260 gm.						
<i>1919</i>	<i>gm.</i>		<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Jan.....	225	4	37.71	19.20	2.79	17.44
Feb.....	186	2	33.34	14.02	2.88	18.00
Mar.....	225	3	35.41	15.83	2.89	18.06
Apr.....	188	2	21.08	0.75	3.03	18.93
May.....	191	5	23.27	2.74	3.11	19.07
June.....						
Dec.....	188	4	40.30	21.38	2.88	18.00
<i>1920</i>						
Jan.....	170	4	37.91	17.89	3.04	19.00
Feb.....	186	17	38.34	18.88	2.89	18.06
Mar.....	186	20	36.53	17.04	2.89	18.06
Apr.....	169	20	27.52	6.67	3.08	19.25
May.....	180	10	25.78	4.00	3.32	20.75
June*.....	170	4	25.22	2.75	3.38	21.13

\* All June analyses were made June 3, as no sardines of this size could be obtained later in the month.

#### DISCUSSION.

There may be a striking variation in the composition of individual sardines as will be seen from a study of Table III. Although the average fat content of the ten sardines analyzed was 0.89 per cent, eight of the ten fish had less than this amount while one, No. 8, had twice the average amount and another, No. 4, had over five times the average. Even when a composite sample is based on as many as ten fish, it has been found that erratic results may be obtained. There is usually a fairly regular increase in fat content with increasing size of the sardine. This increase is illustrated in the analyses of March 29 as shown in Table V which are typical

of fourteen similar series made from January to June, 1920. However, the analyses of April 8, shown in the same table, are quite variable due, no doubt, to the fact that there was great variation in individuals at this time just as there was during the same period of the previous season, as indicated in Table III.

It is apparent from the analyses of small sardines as presented in Table I that there is considerable although sometimes inconsistent, seasonal variation in fat content. The minimum fat percentages in "quarters" was found in October, 1918, and January and February, 1919. Yet the period of maximum fat content extended from July, 1919 to September, 1919. Unfortunately, sardines of these two groups were not available later in the year and so it is impossible to say whether there is always to be expected a sudden decrease in the fat content of small sardines in the fall. It does seem certain that their maximum fat content is reached during the late summer.

It has been shown in a previous paper that mackerel were fatter in the fall of 1919 than in the previous fall. It was also true, as indicated in Tables I and IV, that large sardines were fatter in the season of 1919-20 than in the previous season. This is true month for month in the case of "small ovals" and with the apparent exception of January, 1919, is true of "large ovals" as well. This one exception may be explained by the high average weight of the January, 1919, samples, 225 gm. contrasted with 170 gm., the average weight of the January, 1920, samples.

Fairly consistent variations in the fat contents of large sardines are revealed by a study of Tables I and IV. The fat contents were at a maximum from December to February or March and appeared to drop off rapidly in April of both 1919 and 1920. Evidence was found that this sudden dropping off may have a different explanation from the obvious one. Thus in Table V are shown the analyses of sardines of three different dates during the transition period from high to low fat content. The fish analyzed on March 29 and April 12 were relatively fat while those analyzed at an intermediate date, April 8, were thin. This strongly indicates that there is much difference in schools at this season of the year and suggests that the fish of March 29 and April 12 were from the same or similar schools while those of April 8 were from a school of thin fish.



The opinion has been expressed that the decrease in fat content of the large sardine is closely related to the development of the gonads and the approach of the spawning season. The fact that the time of low fat content and the spawning period are nearly coincident lends support to this view. Considerable attention has

TABLE V.  
*Variation in the Composition of Sardines from Different Schools.*

Description of fish analyzed.				Composition of the edible portion.			
Date.	Average weight per fish.	Weight of gonads divided by total weight.	Average length.	Solids.	Ether extract.	Total nitrogen	Protein (N $\times$ 6.25).
1920	gm.		cm.	per cent	per cent	per cent	per cent
Mar. 29. ....	80	0.037	19.2	25.56	3.20	3.36	21.00
" 29. ....	100	0.036	21.0	25.92	4.20	3.24	20.25
" 29. ....	120	0.031	21.8	29.93	9.11	3.17	19.81
" 29. ....	140	0.058	22.4	34.03	14.09	2.96	18.50
" 29. ....	160	0.052	23.7	34.57	14.78	2.93	18.31
" 29. ....	180	0.054	24.0	35.77	15.89	2.93	18.31
" 29. ....	200	0.044	25.1	35.27	15.06	2.98	18.63
" 29. ....	220	0.048	25.9	35.25	15.11	2.94	18.37
" 29. ....	240	0.048	26.9	33.43	13.39	2.99	18.69
Apr. 8. ....	100	0.028	21.1	23.30	2.13	3.16	19.75
" 8. ....	120	0.025	22.0	23.63	3.23	3.08	19.25
" 8. ....	140	0.033	23.1	24.04	3.38	3.11	19.44
" 8. ....	160	0.043	24.0	23.55	2.65	3.14	19.63
" 8. ....	180	0.027	25.4	21.66	0.88	3.11	19.44
" 8. ....	200	0.038	26.2	23.31	3.42	3.00	18.75
" 8. ....	220	0.042	27.0	27.09	6.25	3.10	19.37
" 12. ....	100	0.049	20.5	29.19	7.89	3.15	19.69
" 12. ....	160	0.082	23.7	31.84	10.95	3.09	19.31

Average of 100 and 160 gm. samples from each group.

Mar. 29. ....	130	0.044	22.3	30.24	9.49	3.08	19.25
Apr. 8. ....	130	0.035	22.5	23.42	2.39	3.15	19.69
" 12. ....	130	0.065	22.1	30.51	9.42	3.12	19.50

been devoted to this question of the relation of percentage of fat to degree of sexual development. In Table VI are shown the analyses of ovaries and testes of the sardine in March, April, and June, 1920. It is evident that there is no great change in the composition of the reproductive organs as spawning season

approaches. The only consistent change is a decreasing fat percentage both in the ovaries and testes.

Table VII shows the changing weight of the gonads as the season advances. The maximum was reached during April or May at which time spent fish began to appear; at this point the relative

TABLE VI.  
*Composition of the Gonads of Sardines.*

No.	Sample.	Date.	Solids.	Ether extract.	P <sub>2</sub> O <sub>5</sub>	Total nitrogen.	Protein (N×6.25).
		1920	per cent	per cent	per cent	per cent	per cent
1	Testes.	Mar. 25	20.84	2.59	1.28	2.80	17.50
2	Ovaries.	" 25	27.66	4.54	1.08	3.24	20.25
3	Testes.	Apr. 12	20.77	2.39	1.21	2.91	18.19
4	Ovaries.	" 12	29.59	3.76	1.17	3.66	22.88
5	Testes.	June 3	20.52	1.07	1.25	2.97	18.56
6	Ovaries.	" 3	23.89	2.12	0.98	3.00	18.75

TABLE VII.  
*Ratio of Weight of Gonads to Total Body Weight.*

No.	Date.	Ratio for "small ovals."	Ratio for "large ovals."
	1920		
1	Jan. 20	0.015	0.018
2	Feb. 2	0.017	0.023
3	" 17	0.022	0.029
4	Mar. 3	0.025	0.028
5	" 10	0.036	0.035
6	" 19	0.047	0.045
7	" 29	0.035	0.051
8	Apr. 8	0.029	0.037
9	" 12	0.049	0.082
10	" 19	0.047	0.067
11	" 27	0.026	0.057
12	May 5	0.046	0.072
13	" 17	0.023	0.046
14	June 3	0.009	0.052

weight of gonads began to decrease as the proportion of spent fish increased. It was found that "small ovals" tend to spawn before the larger sardines. This tendency is shown by the data of June 3 in Table VII. Gonads composed only 0.009 of the total weight of "small ovals" and 0.052 of the weight of "large

ovals." Of the fish analyzed on June 3, 28 out of 30 "small ovals" were spent while only 12 out of 40 "large ovals" were spent. The maximum weight of gonads found was on April 12 (Table VII, No. 9) and was 0.082 of the body weight. The analysis of this particular sample is shown in Table V (160 gm. sample of April 12)—the fat content was relatively high, 10.95 per cent, showing that a considerable growth of the reproductive organs can take place without drawing to any great extent on the reserve store of fat.

TABLE VIII.

*Composition of Sardines of Different Degrees of Sexual Development.*

Description of fish analyzed.				Composition of the edible portion.			
Date.	Average weight per fish.	Weight of gonads divided by total weight.	Average length.	Solids.	Ether extract.	Total nitrogen.	Protein (N×6.25).
1920	gm.		cm.	per cent	per cent	per cent	per cent
Feb. 23 (a)..<	154	0.040	23.3	38.89	18.18	2.92	18.25
" 23 (b)..<	151	0.017	23.0	37.86	18.13	2.93	18.31
" 23 (c)..<	149	0.025	23.2	37.42	17.21	2.96	18.50
" 23 (d)..<	151	0.016	23.3	36.73	16.27	3.03	18.94
Apr. 2 (a)..<	130	0.082		34.22	14.35	2.88	18.00
" 2 (b)..<	124	0.052		32.73	12.58	2.92	18.25
" 2 (c)..<	136	0.044		33.70	14.07	2.92	18.25
" 2 (d)..<	131	0.029		31.52	11.46	2.90	18.13

(a) represents the values found with relatively mature males; (b), relatively immature males; (c), relatively mature females; and (d), relatively immature females.

This same question was approached from another angle. Advantage was taken of the fact that the reproductive organs are of different degrees of maturity in sardines of the same size from the same school. On two occasions fifty or more fish of approximately the same weight were selected and from these ten relatively mature and ten relatively immature fish of each sex were segregated and analyzed. The results, shown in Table VIII, are remarkable for their close similarity. The only consistent variation is that the samples of highest fat content are from the more mature fish. This strongly indicates that in sardines the develop-

ment of the reproductive organs is not closely related to the decreasing fat content although both take place at about the same time.

In some cases the relation between the fat content of fish and the sea temperature has been established. It has been shown, for example, that sea temperature is a factor in the variable fat content of the herring of European waters. It has been found that the minimum sea temperature at the surface off southern California is reached in January or February while the maximum is reached in July or August with a range of 6 or 7°C.<sup>1</sup> Since the fat content of large sardines is near the maximum in January or February, the possibility of more than a remote relationship between these factors in the case of the sardine must be slight.

Aside from the determination of constituents shown in the tables, several determinations of glycogen in sardines have been made. Three of these five determinations yielded 0.50, 0.17, and 0.22 per cent of glycogen in the edible portion. The other two showed no trace of glycogen but, as these two samples were from fish which had been out of the water for several hours while the other three were based on live fish, the absence of glycogen may have been due to hydrolysis. On one occasion several hundred grams of flesh from live sardines were rapidly ground and mixed with a strong potassium hydroxide solution. Instead of hydrolysis of the glycogen and determination of the sugar in the usual way, the glycogen was separated and purified by repeated reprecipitation with alcohol. In this way several decigrams of an amorphous brown powder were obtained which gave an opalescent aqueous solution which produced a red color with iodine.

#### CONCLUSIONS.

Considerable variation in the composition of individual sardines of the same size and from the same school may occur.

Small sardines were found to have a maximum fat content in the summer months.

With some exceptions, other factors being the same, the fat content of sardines increases with the increasing size of the fish.

<sup>1</sup> McEwen, G. F., Summary and interpretation of the hydrographic observations, made by the Scripps Institution for Biological Research of the University of California, 1908 to 1915, Berkeley, 1916.

Marked variations in the fat content of a seasonal nature were found in large sardines, the percentage of fat dropping from a maximum in December or earlier to a minimum in May. This variation was more extreme in 1918-19 than in the following season.

Great difference in the fat content of sardines of the same size from different schools was observed. The migration of schools may be related to the sudden decrease in fat content that takes place in April of each season.

No evidence that the growth of the reproductive organs draws to any great extent on the reserve store of fat was derived.

The relation between the percentage of fat in the sardine and the sea temperature, if any, is remote.

There are appreciable percentages of glycogen in the flesh of the sardine.



## THE ESTIMATION OF CREATININE IN THE PRESENCE OF ACETONE AND DIACETIC ACID.

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(Received for publication, June 27, 1921.)

Since the adaptation by Folin of the Jaffé reaction to the quantitative estimation of creatinine in urine, the procedure has been made the basis of much important research bearing on the problem of the metabolism of creatine and creatinine. The accuracy and reliability of the method have only been questioned in the case of pathological urines, more particularly in specimens containing acetone and diacetic acid. Many of the experiments dealing with this question that were described in the literature offer inconclusive and contradictory evidence as to the exact mode and extent of interferences of the acetone bodies with the determination of creatinine by the Folin method. Klercker<sup>1</sup> stated that large amounts of acetone cause a rapid fading of the creatinine color, while van Hoogenhuyze and Verploegh<sup>2</sup> claimed that the color is at first too dark, but soon fades to a point where it gives correct creatinine reading. Similar results were reported by Rose<sup>3</sup> with regard to the effect of diacetic acid on the color of creatinine and alkaline picrate. His attention was arrested by the assertion of Krause<sup>4</sup> that diacetic acid causes a measurable increase in color, leading ultimately to too low creatine values. From his own investigation, Rose draws the conclusion that diacetic acid, if present in amounts not exceeding 0.25 per cent,

<sup>1</sup> af Klercker, K. O., *Biochem. Z.*, 1907, iii, 45.

<sup>2</sup> van Hoogenhuyze, C. J. C., and Verploegh, H., *Z. physiol. Chem.*, 1908, lvii, 161.

<sup>3</sup> Rose, W. C., *J. Biol. Chem.*, 1912, xii, 73.

<sup>4</sup> Krause, R. A., *Quart. J. Exp. Physiol.*, 1910, iii, 289.

gives an increase in color, which, however, soon fades and offers therefore no serious obstacle to the correct estimation of creatinine. In concentrations larger than 0.25 per cent, the color due to diacetic acid is more persistent and hence objectionable. He furthermore states that acetone in all concentrations is without influence on the reading.

The conclusions with regard to the effect of diacetic acid are far from incontestable, for the reason that Rose used the ethyl ester of diacetic acid and not the acid itself in his experiments. As was shown by Graham and Poulton,<sup>5</sup> and we can confirm their findings, these two substances do not behave in an analogous manner with respect to their effect on the color reaction of creatinine. The same criticism may be applied to the work of Wolf and Osterberg,<sup>6</sup> who found that 1.0 per cent of ethyl acetoacetate caused no marked change in the creatinine reading.

In contrast to the results of Rose stand the reported findings of Greenwald<sup>7</sup> that acetone in amounts greater than 0.5 per cent gives at first an undue increase in color, which soon drops below its normal value on account of fading. In the presence of diacetic acid the creatinine color is always too light. Greenwald estimated the amount of diacetic acid added to urine by the intensity of the ferric chloride reaction and made no attempt to determine the minimum amount of diacetic acid which may possibly interfere. Graham and Poulton<sup>5</sup> detail careful experiments in which they have added graded quantities of acetone, ethyl acetoacetate, and sodium acetoacetate to urine and studied the effect of these substances on the creatinine color. They concluded that acetone, if less than 0.2 per cent, causes no error; with larger amounts the color is lighter than normal. Ethyl acetoacetate in small amounts (0.1 to 0.7 per cent) gives a lighter color; larger amounts give a darker color which increases on standing. Sodium acetoacetate, even in small amounts, causes a lighter color; with larger amounts the color is still lighter and fades rapidly on standing. Consequent upon these findings, they have investigated the question of alleged creatinuria in carbohydrate starvation. As a result of

<sup>5</sup> Graham, G., and Poulton, E. P., *Proc. Roy. Soc. London, Series B*, 1913-14, lxxxvii, 205.

<sup>6</sup> Wolf, C. G. L., and Osterberg, E., *Am. J. Physiol.*, 1911, xxviii, 71.

<sup>7</sup> Greenwald, I., *J. Biol. Chem.*, 1913, xiv, 87.



their study, they have come to the conclusion that the figures for urine creatine in carbohydrate starvation reported by previous observers merely represented discrepancies between correct and incorrect creatinine determinations, due to the respective absence and presence of diacetic acid in the urine after and before heating of the specimen for the conversion of creatine to creatinine.

In view of these conflicting statements with regard to the effect of acetone and diacetic acid on the estimation of creatinine and their bearing on the problem of creatine metabolism, as pointed out by Graham and Poulton, it was deemed worth while to subject the matter to a critical experimental examination. We have studied the effect of added acetone, diacetic acid, and ethyl acetoacetate on the color reaction of creatinine in pure solution and in urine.

*Effect of Acetone on the Color Reaction of Creatinine in Pure Solution.*—A stock solution of pure creatinine was made by dissolving 1.0 gm. in 1,000 cc. of 0.1 N HCl (1.0 cc. = 1.0 mg. of creatinine), 2 and 5 cc. portions, respectively, were placed in a 500 cc. volumetric flask, acetone and water were added to a volume of 10 cc. The solution was then treated with 15 cc. of saturated picric acid and 5 cc. of 10 per cent sodium hydroxide solution, allowed to stand for 8 to 10 minutes, and after diluting to the mark, compared with a standard creatinine solution similarly treated with picric acid and alkali. The results are given in Table I.

*Effect of Added Acetone on the Determination of Creatinine in Urine.*—A quantity of urine was boiled in an open flask for about 10 minutes to remove all traces of acetone and diacetic acid. It was then cooled to room temperature and its creatinine content estimated, using a 0.5 N  $\text{K}_2\text{Cr}_2\text{O}_7$  solution as a standard. Different portions of the sample were mixed with varying amounts of acetone and each analyzed for creatinine (Table II).

Tables III and IV give the results of experiments with ethyl acetoacetate.

*Experiments with Diacetic Acid and Acetone.*—A pure solution of diacetic acid was prepared by the hydrolysis of its ethyl ester according to the directions of Ceresole.<sup>8</sup> Just before using, the solution was placed in a tall cylinder and a current of acetone-free

<sup>8</sup> Ceresole, M., *Ber. chem. Ges.*, 1882, xv, 1871.

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air slowly drawn through it for about 45 minutes to remove traces of acetone that have been formed from the spontaneous decomposition of the acid. A measured volume of it was then transferred to a Kjeldahl flask, diluted with several volumes of water, acidified with  $\text{H}_2\text{SO}_4$ , and distilled into water. The acetone caught in the

TABLE I.

*Effect of Acetone on the Color Reaction of Creatinine in Alkaline Picrate.*

Creatinine taken.	Acetone added to 10 cc.		Color.	Creatinine found.	Error.
mg.	mg.	per cent	mm.	mg.	per cent
2.0	0.0	0.0	10.0	2.0	0.0
2.0	20.0	0.20	10.0	2.0	0.0
2.0	40.0	0.40	10.1	1.98	- 1.0
2.0	50.0	0.50	10.1	1.98	- 1.0
2.0	60.0	0.60	10.3	1.94	- 3.0
2.0	70.0	0.70	10.6	1.88	- 6.0
2.0	80.0	0.80	10.8	1.85	- 7.5
2.0	100.0	1.00	11.5	1.74	-13.0
2.0	150.0	1.50	12.8	1.55	-22.0
2.0*	200.0	2.0	15.7	1.27	36.5
5.0	0.0	0.0	10.0	5.0	0.0
5.0	40.0	0.40	10.0	5.0	0.0
5.0	50.0	0.50	10.0	5.0	0.0
5.0	60.0	0.60	10.1	4.95	- 1.0
5.0	70.0	0.70	10.3	4.85	- 3.0
5.0	90.0	0.90	10.5	4.76	- 4.8
5.0	120.0	1.20	11.0	4.54	- 9.2
5.0	180.0	1.8	11.7	4.27	-14.6
5.0	500.0	5.0	14.0	3.57	-28.6
5.0†	800.0	8.0	33.0	1.51	-65.8

\* Standard, 2.0 mg. of pure creatinine set at 10 mm.

† Standard, 5.0 mg. of pure creatinine set at 10 mm.

receiver was then determined by the Messinger titration. From the figures obtained, the percentage of diacetic acid in the solution was calculated. Definite quantities of this solution were added to known amounts of creatinine in pure solution, and in urine, and their effect noted. The results are summarized in Tables V and VI.

## DISCUSSION.

The data in the tables show consistently that, barring a few minor exceptions, our results are in substantial agreement with those obtained by Graham and Poulton. Acetone in large amounts undoubtedly fades the creatinine color from the very outset. We are inclined to set the upper limit of allowable acetone concentration at 0.50 per cent. The figures presented by Graham and

TABLE II.

*Effect of Acetone on the Determination of Creatinine in Urine.\**

Acetone added to 10 cc. of urine.		Color.	Creatinine in 100 cc. of urine.	Error.
<i>mg.</i>	<i>per cent</i>	<i>mm.</i>	<i>mg.</i>	<i>per cent</i>
0.0	0.0	7.7	105.19	0.0
40.0	0.4	7.7	105.19	0.0
50.0	0.5	7.7	105.19	0.0
60.0	0.6	7.8	103.87	- 1.19
70.0	0.7	7.9	102.53	- 2.52
90.0	0.9	8.4	96.42	- 8.35
120.0	1.2	8.8	92.04	-12.46
150.0	1.5	9.1	89.01	-15.38
200.0	2.0	9.8	82.65	-21.42
0.0	0.0	8.1	100.00	0.0
30.0	0.3	8.1	100.00	0.0
50.0	0.5	8.2	98.78	- 1.22
60.0	0.6	8.1	100.00	0.0
70.0	0.7	8.4	97.62	- 2.38
80.0	0.8	8.6	94.18	- 5.82
90.0	0.9	8.7	93.0	- 7.00
100.0	1.0	9.0	90.0	-10.00
150.0	1.5	13.5	60.0	-40.00
300.0	3.0	16.0	50.62	-49.38

\* Standard, 0.5 N potassium dichromate. All tests, giving higher reading, fade on standing.

Poulton show that 0.17 per cent acetone had no effect on the reading, but 1.0 per cent gave a markedly lighter color. The gap between 0.17 and 1.0 per cent is obviously a wide one. We can say that the fading effect of acetone does not begin markedly to show itself until a concentration of 0.70 per cent has been reached. With no amount of acetone did we observe an increase in color (Tables I and II).

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TABLE III.  
*Effect of Ethyl Acetoacetate on Color of Pure Creatinine.\**

Creatinine taken.	Ester added to 10 cc.		Color.	Creatinine found.	Error.
mg.	mg.	per cent	mm.	mg.	per cent
5.0	0.0	0.0	10.0	5.0	0.00
5.0	5.0	0.05	10.0	5.0	0.00
5.0	10.0	0.10	10.1	4.95	- 1.00
5.0	20.0	0.20	10.3	4.85	- 3.00
5.0	50.0	0.50	9.8	5.10	+ 2.00†
5.0	60.0	0.60	9.0	5.55	+11.00†
5.0	100.0	1.00	8.0	6.25	+25.00†

\* Standard, 5 mg. of pure creatinine set at 10 mm.

† Did not fade after standing for 10 minutes.

TABLE IV.  
*Effect of Ethyl Acetoacetate on Determination of Creatinine in Urine.\**

Ester added to 10 cc.		Color.	Creatinine in 100 cc. of urine.	Error.
mg.	per cent	mm.	mg.	per cent
0.0	0.0	12.2	66.39	0.00
10.0	0.10	12.5	64.80	- 2.39
20.0	0.20	12.6	64.28	- 3.17
50.0	0.50	12.4	65.32	- 1.61
60.0	0.6	12.0	67.5†	+ 1.67†
100.0	1.0	11.0	73.63†	+10.90†
200.0	2.0	9.0	90.0†	+35.56†

\* Standard, 0.5 N potassium dichromate.

† No fading, but slight increase of color after 10 minutes.

TABLE V.  
*Effect of Diacetic Acid on Color of Pure Creatinine in Alkaline Picrate.\**

Creatinine taken.	Diacetic acid : 10 cc.		Color.	Creatinine found.	Error.
mg.	mg.	per cent	mm.	mg.	per cent
5.0	0.00	0.00	10.0	5.00	0.00
5.0	1.5	0.015	10.0†	5.00	0.00
5.0	2.1	0.021	10.3	4.85	- 3.00
5.0	3.0	0.030	10.7	4.67	- 6.60
5.0	4.5	0.045	11.2	4.46	-10.80
5.0	9.0	0.09	12.3	4.06	-18.80
5.0	15.0	0.15	13.5	3.70	-26.00

\* Standard, 5 mg. of pure creatinine treated the same as the unknown.

† Fades after about 5 minutes to 10.2. All other tests fade even more rapidly.

Ethyl acetoacetate in small amounts causes a fading in the creatinine color, while in larger amounts it causes an increase in color. The line of demarkation is difficult to establish, as the two

TABLE VI.

*Effect of Diacetic Acid on the Determination of Creatinine in Urine.*

Acid added to 10 cc.		Color.	Creatinine in 100 cc.	Error.
<i>mg.</i>	<i>per cent</i>	<i>mm.</i>	<i>mg.</i>	<i>per cent</i>
0.0	0.0	3.0	270.00	0.00
2.1	0.021	3.1	261.29	- 3.22
3.0	0.030	3.2	253.12	- 6.25
4.5	0.045	3.4	238.23	-12.13
6.0	0.06	3.8	213.16	-21.05
9.0	0.09	4.1	197.56	-26.82
15.0	0.150	5.5	147.27	-45.45
0.0	0.0	5.1	158.82	0.00
2.1	0.021	5.3	152.83	- 3.51
3.0	0.030	5.5	147.27	- 7.27
4.5	0.045	5.7	142.10	-10.52
6.0	0.060	6.1	132.78	-16.39
9.0	0.090	6.9	117.39	-26.08
15.0	0.150	8.2	98.78	-37.80
0.0	0.0	6.8	119.11	0.00
1.107	0.011	7.0	115.71	- 2.85
2.214	0.022	7.3	110.16	- 7.51
3.321	0.033	7.7	105.19	-11.68
5.535	0.55	8.4	96.42	-19.05
0.0	0.0	8.3	98.79	0.00
2.77	0.027	9.8	82.55	-16.42
5.535	0.055	10.8	75.00	-24.08
11.07	0.110	13.0	62.30	-36.93
0.0	0.0	12.5	64.80	0.00
1.107	0.011	13.0	62.30	- 3.83
2.214	0.022	13.3	60.90	- 6.01
4.428	0.044	15.0	54.00	-16.66
5.535	0.055	16.7	48.50	-25.15

limits seem to merge into each other by an almost insensible gradation (Tables III and IV). This is, of course, of no great importance, since diacetic acid does not occur in urine in the form of its ethyl ester.

Our experiments with diacetic acid bear out the conclusion of Graham and Poulton that this substance, even in small amounts, has a great influence on the creatinine reading. 0.015 or 0.02 per cent causes a perceptible fading, and the color becomes progressively lighter, as the concentration of the acid or the time for which the test is allowed to stand is increased (Tables V and VI).

The tables show uniformly that the influence of a given amount of acetone or diacetic acid on the determination of creatinine is more marked in dilute solutions than in more concentrated ones, and also that the effect is the same, whether the creatinine is in a pure solution or in urine.

The above findings point clearly to the necessity of removing diacetic acid from urine, preparatory to the determination of creatinine in the sample. Of the efforts that have been made towards the development of a suitable technique to serve this purpose, worthy of note are those of Greenwald and of Graham and Poulton.<sup>5</sup> Following the suggestion of Rona, Greenwald has attempted to drive off diacetic acid from urine by boiling, but, after a few trials with urines from cases of muscular dystrophy, abandoned the method because it invariably resulted in higher creatinine values. Whether this was due to the expulsion of diacetic acid, and was therefore the desired result, to the conversion of creatine to creatinine or to the undue concentration of the urine leading to the formation of pigments and chromogenic condensation products; whether any or all of these factors were operative in the production of a greater intensity of color, Greenwald did not ascertain. He adopted the procedure of extracting the urines with ether in a continuous extractor for 2 hours, blowing off the ether, diluting the cool solution to twice its original volume, and developing the creatinine color by the addition of 30 cc. of picric acid and 10 cc. of NaOH. Needless to say that this method is impractical where a great many determinations have to be made. It is tedious and time consuming, and is further limited in its usefulness by the fact that it requires expensive apparatus. The same objections apply, though perhaps with not such great force, to the method described by Graham and Poulton. They direct adding 1.0 cc. of a 10 per cent solution of  $H_3PO_4$  to a measured volume of urine and removing the diacetic acid by distillation at  $70^\circ C.$  and 210 mm. for 45 minutes. Then they neutralize with

NaOH, dilute to a definite volume, and determine the creatinine in an aliquot. Besides being complicated, the method yields satisfactory results only when experimental conditions, as prescribed by Graham and Poulton, are strictly adhered to. Otherwise, there is either an incomplete removal of diacetic acid, or a conversion of creatine to creatinine. At best the danger of error from this latter source is great, because of the employment of a strong acid. Results are also apt to be irregular with urines containing sugar. The method thus makes unusual demands on the time and judgment of the analyst.

In order to circumvent these difficulties we have set about to find a new method for removing diacetic acid from urine. We have tried first to boil urine with water. Unlike Greenwald, we have found that most urines may be thus boiled without causing an appreciable increase in the creatinine figure. Our procedure was as follows:

10 cc. of urine were measured into a 300 cc. flask or beaker, 3 to 4 volumes of water and 5 to 10 mg. of creatine were added, and the liquid was boiled down to its original volume in 5 to 8 minutes. The solution was then cooled in running water, mixed with 15 cc. of saturated picric acid and 5 cc. of 10 per cent NaOH. After 10 minutes the colored solution was transferred quantitatively to a 500 cc. volumetric flask, diluted to the mark, and read in the colorimeter. We have applied the method to a number of urines from a variety of hospital cases. As may be seen from Table VII, a markedly increased creatinine color in boiled urine is an exception rather than the rule.

This increase in color is presumably due to the conversion of creatine to creatinine. That this is not the sole factor is apparent from the fact that when urines containing no creatine at all are boiled, a slightly darker color may result. Again, many specimens containing added creatine may be boiled without any effect on the color. That the conversion of creatine may be insignificant as compared with positive errors from other sources may be shown by the following experiment:

At a given temperature, the rate of conversion of a definite amount of creatine is conditioned by the hydrogen ion concentration of the medium. Now, when the pH is decreased by the addition of sodium acetate, it is reasonable to suppose that the rate of

TABLE VII.

*Effect of Boiling Urine on the Determination of Creatinine.*

No.	Creatine added to 10 cc.	Unboiled urine.		Boiled urine.		Error.	Remarks.
		Color	Creatinine.	Color	Creatinine.		
	mg.	mm.	mg.	mm.	mg.	per cent	
1	0.0	8.5	95.29	8.5	95.29	0.0	Normal.
2	0.0	9.7	83.50	9.8	82.65	-1.01	"
3	0.0	5.0	162.00	5.0	162.00	0.0	"
4	0.0	8.7	93.10	8.5	95.29	0.0	"
5	0.0	7.6	106.57	7.5	108.00	+1.34	Exophthalmic; goiter; creatine, 233 mg.
6	0.0	4.5	180.00	4.5	180.00	0.0	Diabetes; no sugar or diacetic acid.
7	0.0	5.7	142.10	5.8	139.62	-1.74	Active pulmonary tuberculosis; creatine, 119 mg.
8	0.0	7.0	115.71	6.8	119.12	+2.94	Acute nephritis.
9	0.0	8.0	101.25	7.8	103.84	+2.55	Hyperthyroidism; creatine, 110 mg.
10	0.0	10.7	75.70	10.4	77.88	+2.86	Chronic nephritis.
11	0.0	12.4	65.35	12.3	65.85	+0.76	Normal.
12	0.0	6.4	126.56	6.7	120.89	-4.48	Normal; initial reaction, alkaline.
13	10.0	10.2	79.41	9.8	82.65	+4.08	Normal.
14	10.0	4.5	180.00	4.5	180.00	0.0	"
15	10.0	6.9	117.29	6.9	117.29	0.0	"
16	10.0	6.4	126.56	6.4	126.56	0.0	"
17	10.0	7.9	102.54	7.4	109.46	+6.75	"
18	10.0	9.2	88.04	9.0	90.00	+2.23	Lues.
19	10.0	9.0	90.00	9.0	90.00	0.0	Pneumonia.
20	10.0	12.6	64.28	12.2	66.39	+3.28	Diabetes.
21	10.0	10.5	77.14	10.4	77.88	+0.95	Auricular fibrillation.
22	10.0	13.2	61.37	12.6	64.28	+4.57	Pneumonia.
23	10.0	7.0	115.71	6.7	120.89	+4.47	"
24	10.0	12.6	64.28	11.2	72.32	+12.50	Cerebrospinal syphilis.
25	10.0	5.5	147.27	5.5	147.27	0.0	Pernicious anemia.
26	10.0	6.7	120.89	6.7	120.89	0.0	" "
27	10.0	8	101.25	7.3	110.95	9.52	Bronchitis.
28	10.0	5.6	144.64	5.6	144.64	0.0	Tuberculosis.

Creatinine values are for 100 cc. of urine.



conversion of creatine will be retarded, yet the reading of a urine that yields a higher creatinine value on boiling will be the same whether the sample has been boiled for the same length of time with or without the buffer. It is evident then that some other element besides the change of creatine into creatinine enters into the production of a greater intensity of color. The fact that normal urines (diacetic acid and creatine-free) to which glucose has been added yield a considerable increase of color after boiling (Tables VIII and IX) would seem to indicate that condensation products play an important rôle in the process. In working with dog urines the fact has also developed that during boiling the reaction often becomes less acid or even slightly alkaline, in consequence of which there is a slight loss of creatinine.

Because of these occasional irregularities of the creatinine readings, encountered when urines are boiled with water, we do not feel justified in recommending the procedure for general adoption. A much safer method consists in boiling the urine after the addition of a substance which lowers the boiling point and at the same time has no effect on either creatine or creatinine. Methyl alcohol has been found to answer these requirements. It is also relatively inexpensive and readily obtainable.<sup>9</sup>

To show that methyl alcohol does not change the composition or properties of creatine and creatinine, a mixture of an aqueous solution of these two compounds was slowly boiled over an asbestos mat with about 5 volumes of the reagent until the temperature reached 100°C. The solution was then cooled, picric acid and alkali were added, and the color was developed in the usual way. The correct reading for the creatinine content of the solution was obtained. The same result was obtained when the solution was slightly acidified with lactic or acetic acid and treated as just described. Pure solutions, as well as urines to which excessive quantities of diacetic acid were added, were found, after this treatment, to be negative to the ferric chloride reaction. The legal reaction occasionally revealed the presence of traces of acetone, but these had no measurable influence on the creatinine reading. Urines containing added glucose (Tables VIII and IX) were

<sup>9</sup> The use of methyl alcohol in this connection was suggested by Dr. S. R. Benedict, to whom the writer is indebted for much helpful advice during the course of this work.

TABLE VIII.

*Experiments with Methyl Alcohol Boiling to Remove Diacetic Acid from Urine.*

No.	Unboiled.		Acid added.		Creatine added.	Boiled + alcohol.		Error.
	Color.	Creatinine.				Color.	Creatinine.	
	mm.	mg.	mg.	per cent	mg.	mm.	mg.	per cent
1	11.7	69.23	11.07	0.1107	10.0	11.7	69.23	0.0
2	7.2	112.50	11.07	0.1107	10.0	7.2	112.50	0.0
3	7.7	105.19	11.07	0.1107	10.0	7.7	105.19	0.0
4	7.2	112.50	11.07	0.1107	10.0	7.2	112.50	0.0
5	5.2	155.76	11.07	0.1107	10.0	5.2	155.76	0.0
6	10.3	78.64	11.07	0.1107	10.0	10.3	78.64	0.0
7	8.0	101.25	11.07	0.1107	10.0	8.0	101.25	0.0
8	6.8	119.11	11.07	0.1107	10.0	6.8	119.11	0.0
9	8.0	101.25	11.07	0.1107	10.0	8.0	101.25	0.0
10	5.7	142.10	11.07	0.1107	10.0	5.8	139.62	-1.74
11	10.4	77.80	11.07	0.1107	10.0	10.4	77.80	0.0
12	12.8	63.51	11.07	0.1107	10.0	12.8	63.51	0.0
13	8.7	93.1	11.07	0.1107	10.0	8.7	93.1	0.0
14	6.4	126.56	11.07	0.1107	10.0	6.4	126.56	0.0
15	12.8	63.51	11.07	0.1107	10.0	12.8	63.51	0.0
16	15.0	54.00	11.07	0.1107	10.0	15.0	54.00	0.0
17	12.5	64.80	11.07	0.1107	10.0	12.5	64.80	0.0
18	5.8	139.62	11.07	0.1107	10.0	5.9	137.28	-1.60
19	9.4	86.16	11.07	0.1107	10.0	9.4	86.16	0.0
20	5.9	137.28	11.07	0.1107	10.0	5.8	139.62	+1.70
21	8.3	97.59	0.0	0.0	0.0	8.3	97.59	0.0
21	8.3	97.59	2.77	0.0277	2.0	8.3	97.59	0.0
21	8.3	97.59	5.53	0.0553	2.0	8.3	97.59	0.0
22	7.5	108.00	0.0	0.0	2.0	7.5	108.00	0.0
23	12.5	64.80	1.107	0.0117	2.0	12.5	64.80	0.0
23	12.5	64.80	2.214	0.02214	2.0	12.5	64.80	0.0
23	12.5	64.80	4.43	0.0443	2.0	12.5	64.80	0.0
23	12.5	64.80	5.53	0.0553	2.0	12.5	64.80	0.0
24	7.7	105.19	70.40	0.704	8.0	7.7	105.19	0.0
25	8.3	97.59	70.40	0.704	8.0	8.3	97.59	0.0
26	8.0	101.25	70.40	0.704	8.0	8.0	101.25	0.0
27	9.0	90.0	70.40	0.704	8.0	9.0	90.0	0.0

Creatinine values are for 100 cc. of urine.

but very slightly darker than normal after having been boiled with methyl alcohol, and gave correct creatinine readings; while the same urine boiled with water gave a darker color with a brownish tint, which made colorimetric comparison somewhat difficult.

TABLE IX.  
*Experiments with Dog Urines.*

No.	Unboiled.		Boiled + alcohol.		Error.	Boiled + water.		Error.	Graham and Poulton distillation.		Error.
	Read- ing.	Creati- nine.	Read- ing.	Creati- nine.		Read- ing.	Creati- nine.		Read- ing.	Creati- nine.	
	mm.	mg.	mm.	mg.	per cent	mm.	mg.	per cent	mm.	mg.	per cent
28	8.7	93.10	8.7	93.10	0.0						
29	12.6	64.28	12.6	64.28	0.0				10.5	77.14	+20.00
30	8.9	91.01	8.9	91.01	0.0	9.1	89.01	- 2.19	12.0	67.5	-24.72
31	9.1	89.01	9.1	89.01	0.0	8.6	93.07	+ 4.56	9.1	89.01	0.0
32	12.2	66.39	12.2	66.39	0.0	12.0	67.50	+ 1.67	11.0	73.63	+10.90
33	11.8	68.64	11.8	68.64	0.0	12.2	66.48	- 1.68	11.8	68.64	0.0
34	9.0	90.00	9.1	89.01	-1.1	8.9	91.01	+ 1.12	8.7	93.10	+ 7.66
35	9.9	81.81	9.9	81.81	0.0	10.0	81.00	- 0.99	9.6	84.37	+ 3.12
36	10.2*	79.41	10.2	79.41	0.0	8.0	101.25	+21.52	8.3	97.59	+22.89
37	8.0†	101.25	8.0	101.25	0.0	7.5	108.00	+ 6.66	8.7	93.10	-14.71
38	8.8†	92.45	8.8	92.45	0.0	8.6	93.07	+ 0.67	7.6	106.57	+15.27

\* 10.0 per cent of glucose added.

† 5.0 " " " " "

Diacetic acid added to each sample = 70.40 mg. to 10.0 cc. = 0.704 per cent.

Creatine added to each sample = 5.0 mg. to 10.0 cc.

Values are mg. per 100.0 cc. of urine.

Standard = 0.5 N dichromate.

The details of the proposed method are as follows: 10 cc. of urine are introduced into a 300 cc. Erlenmeyer or round bottom flask containing a few glass beads. If the urine is alkaline the reaction should be adjusted with strong HCl to be that due to weak organic acids; that is, it should be red to litmus but not blue to Congo red. 5 volumes of methyl alcohol are added and the mixture is slowly boiled over an asbestos mat until 1 or 2 minutes

after the temperature has risen to  $100^{\circ}\text{C}$ .<sup>10</sup> This should take not less than 15 minutes. The flask is now thoroughly cooled in running water, its contents are mixed with 15 cc. of saturated picric acid and 5 cc. of 10 per cent NaOH, at the end of 8 minutes washed quantitatively into a 500 cc. flask, diluted to mark, and read.

The time allowed for decomposing the diacetic acid and driving off the acetone formed is ample enough for such concentrations of the interfering substance as are ordinarily met with in pathological urines. We have repeatedly freed 10 cc. of urine from as much as 70.4 mg. (0.704 per cent) of added diacetic acid by this method. But for this it is necessary to keep the liquid in a state of active boiling during the entire period of 15 minutes. The mixture usually begins to boil around  $75^{\circ}\text{C}$ . and stays at that temperature for about 8 minutes. After that the temperature gradually rises to  $100^{\circ}$ , and the contents of the flask will have returned to their original volume. Boiling a little longer beyond this point does no harm and rather insures the expulsion of traces of acetone. At first we have tried boiling on a water bath. Under these conditions the temperature can be kept constant at about  $85^{\circ}\text{C}$ . for a considerable length of time, but this was found unnecessary. In several instances, when heating in this way was unduly prolonged, an appreciable increase in the creatinine figure was obtained.

One other precaution is necessary; that is to make sure that the urine is thoroughly cooled before adding the picric acid and the sodium hydroxide. Otherwise, an unduly dark color will result.

Table VIII shows some results obtained by the method.

#### SUMMARY.

The influence of acetone, ethyl acetoacetate, and diacetic acid on the color reaction of creatinine in alkaline picrate was studied. It was found that these substances interfere with the colorimetric determination of creatinine in pure solution as well as in urine.

A method for removing diacetic acid from urine has been described.

<sup>10</sup> The methyl alcohol was usually distilled off through a Liebig condenser. If the alcohol is boiled off from an open vessel the operation should be carried out in a hood on account of the toxicity of methyl alcohol vapors. The recovered methyl alcohol can be readily purified by refluxing with acid mercuric sulfate, filtering off the precipitate formed, and fractionating the filtrate.

# ON THE STRUCTURE OF THYMUS NUCLEIC ACID AND ON ITS POSSIBLE BEARING ON THE STRUCTURE OF PLANT NUCLEIC ACID.

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(Received for publication, July 1, 1921.)

The polynucleotide structure of both yeast and animal nucleic acids is generally accepted; the question of the mode of linking of individual nucleotides continues to be the subject of discussion and of disagreement between individual workers. Thannhauser, Jones, and the present writer, have each put forth a different theory of the structure of the plant nucleic acid. Each author has criticized the theories of the other two. As a result of these criticisms, Thannhauser<sup>1</sup> has modified his theory and incorporated in his formulation the views of Jones. Thus the criticism of the present author of the theory of Jones applies also to that of Thannhauser. It is peculiar that Jones, in the latest edition of his monograph, in discussing the theories of the structure of yeast nucleic acid does not at all refer to the theory of the present writer.

The point of contention is the following: Are the nucleotides united one to another in an ether linking through their carbohydrates, or in ester form, the phosphoric acid of one combining with the carbohydrates of the other. Jones, and with him Thannhauser, accepts the ether linking, the present writer the ester linking. Since the original evidence on which Jones and Thannhauser have based their theories was proved an experimental error, Jones<sup>2</sup> has furnished two new experiments in support of his theory. The first is the following:

The curve expressing the rate of hydrolysis of yeast nucleic acid is identical with that of a mixture of the four nucleotides. Accepting the experiment as correct, what does it demonstrate? It proves that the union between individual nucleotides is more

<sup>1</sup> Thannhauser, S. J., and Sachs, P., *Z. physiol. Chem.*, 1920-21, cii, 187.

<sup>2</sup> Jones, W., *Am. J. Physiol.*, 1920, lii, 193, 203.

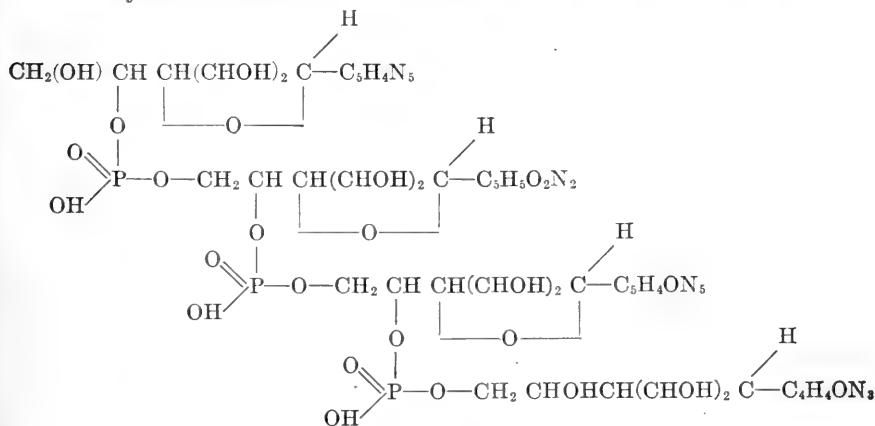
labile than that between the phosphoric acid and the carbohydrate in each nucleotide. It is then self-evident that the first step in the hydrolysis of the nucleic acid molecule is the formation of four nucleotides. The further progress of hydrolysis of the nucleic acid is the same as of four nucleotides. This ready formation of four nucleotides as the initial phase is hard to reconcile with the assumption of Jones of an ether linking between individual nucleotides. On the other hand, this observation is consistent with the theory of the present writer. Truly, the cleavage of nucleic acid into mononucleotides should have been impossible if it were otherwise. The view of Levene is substantiated by the observations of Levene, Meyer, and Yamagawa<sup>3</sup> who found that the rate of hydrolysis of phosphoric acid is practically identical whether 1-2-acetone-3- or 5-phosphoric acid-6-benzoyl glucose, or 1-2-acetone-3- or 5-phosphoric acid glucose are hydrolyzed.

The second experimental proof of Jones' theory is the following: By a pancreas enzyme, yeast nucleic acid was cleaved to its nucleotides. At the starting point of the experiment the hydrogen ion concentration of the reacting mixture was brought to  $\text{pH} = 6.4$ , and at the end of the experiment there was no apparent change of the color of the indicator added to the original solution. Hence the author concludes that no acid radicles could be liberated as the result of the hydrolysis. The reasoning is not correct. According to either theory, nucleic acid is a polyphosphoric acid and when brought to a  $\text{pH} = 6.4$ , it possesses considerable buffer effect. Furthermore, each nucleotide is a comparatively weak acid and when liberated does not affect the hydrogen ion concentration of the buffer very markedly. Since the dissociation constant of the nucleotides has not been measured, it is not possible to express the reaction in quantitative terms. Experimentally, however, we convinced ourselves that when a solution of guanosinphosphoric acid is brought to a  $\text{pH} = 6.4$ , it stands the addition of an equal volume of a solution of free guanosinphosphoric acid of the same concentration before any change of color of the indicator can be noticed. Taking further into consideration the fact that a solution of nucleic acid is not perfectly colorless, that an extract of the pancreas always contains a considerable quantity of phosphates and also is not colorless, one easily realizes that the argument of Jones carries but little weight.

<sup>3</sup> Levene, P. A., and Yamagawa, M., *J. Biol. Chem.*, 1920, xlii, 323. Levene, P. A., and Meyer, G. M., *J. Biol. Chem.*, 1921, xlvi, 233.

Thus, to the mind of the present writer, there exists neither experimental nor theoretical evidence in favor of the theory of the ether linking between individual nucleotides.

On the other hand, the theory of the present writer brings the structure of the yeast nucleic acid in harmony with that of thymus nucleic acid. Levene and Jacobs<sup>4</sup> have described hexothymidindiphosphoric acid and hexocytidindiphosphoric acid obtained on hydrolysis of thymus nucleic acid. They have also isolated hexothymidinmonophosphoric and hexocytidinmonophosphoric acids, and a substance which seemed to them to be a dinucleotide of hexothymidin and hexocytidin. In view of the experience on yeast nucleic acid, it seemed urgent to reinvestigate the question of the dinucleotide. For this purpose larger quantities of the material were required and hence it was attempted to simplify the method of its preparation. The complicated process employed in the older work was abandoned. From the hydrolyzed material the diphosphonucleotides were removed as barium or calcium salt on heating; from the mother liquor of these it was hoped to isolate the monophosphoric nucleotides and the hypothetical dinucleotide. However, under these conditions only the diphosphoric nucleotides could be isolated. It is possible that the monophosphoric acid nucleotides are secondary products, and that the hypothetical dinucleotide is only a mixture of mononucleotides. Thus for the present, until the existence of the dinucleotide is definitely proved, the structure of the thymus nucleic acid should be expressed in analogy with the yeast nucleic acid, namely as follows:



<sup>4</sup> Levene, P. A., and Jacobs, W. A., *J. Biol. Chem.*, 1912, xii, 411.

Incidentally the barium salt of the hexothymidindiphosphoric acid was obtained in crystalline form.

For the final solution of the problem of the structure of thymus nucleic acid larger quantities of the material are needed. Since the manufacture of this nucleic acid in Europe has been discontinued, it will have to be prepared with our laboratory facilities. Because of this, the progress of the work will be delayed, but work is now in progress.

#### EXPERIMENTAL.

Commercial animal nucleic acid (Merck) was hydrolyzed in 100 gm. lots. This amount of the acid was heated on flame with reflux condenser for 2 hours with 1,000 cc. of 2 per cent sulfuric acid. The sulfuric acid and the free phosphoric acid are removed by a slight excess of barium hydroxide solution. The excess of this reagent was then removed quantitatively and the solution concentrated under diminished pressure at room temperature to a volume of 300 cc. The nucleotides were then precipitated with a 25 per cent solution of neutral lead acetate. The precipitate was washed repeatedly with cold water and then suspended in water, decomposed by means of hydrogen sulfide gas. The filtrate from the lead sulfide was again concentrated under diminished pressure at room temperature to a volume of 300 cc. This was again neutralized with barium hydroxide, filtered from the slight trace of barium phosphate, and brought to a boil over a free flame. A flocculent precipitate soon appears, which on prolonged boiling assumes a granular character. The filtrate from this precipitate was then concentrated to small volume and again heated as before; generally a second precipitate formed. The material which formed on boiling (Precipitate I) had the composition of the diphosphonucleotides.

The mother liquor from the diphosphonucleotides was precipitated by alcohol (Precipitate II). This precipitate had the elementary composition of monophosphonucleotides. However, when freed from barium and purified through conversion into lead salt and reconversion with barium salt again, there is formed a barium salt insoluble in boiling water. Thus finally, practically all is converted into the diphosphonucleotides.



There seems to be a discrepancy between the present result and that obtained by Levene and Jacobs. Two alternative explanations may be given to the discrepancy; either the monophosphonucleotides found previously by Levene and Mandel and by Levene and Jacobs are products of further decomposition of the diphosphonucleotides formed in the course of further manipulation, or the monophosphonucleotides are missed in the present procedure.

*Composition of Crude Barium Salts.*

No. of sample.	P	N
358 20/21	8.82	5.36
359 20/21	8.27	5.14
361 20/21	7.74	5.89
366 20/21	7.39	5.62
370 20/21	8.27	5.31
362 20/21	5.66	5.96
474 20/21	5.15	6.11

Samples 362 and 474 were combined, freed from barium, converted into lead salt, and this reconverted into barium salt. The greater part settled out on boiling and had the following composition.

No. of sample.	P	N
408 20/21	7.17	5.50

For further purification and for the separation of individual diphosphonucleotides the older procedure was modified. The barium salts were converted into lead salts and these into brucine salts. The brucine salts were fractionated by recrystallization from 35 per cent alcohol until the more insoluble fraction had the elementary composition of the hexothymidindiphosphoric brucine salt. The combined mother liquors were then concentrated and allowed to stand until a crystalline deposit formed. This was again refractionated. After two refractionations the more soluble brucine salt on conversion into the barium salt analyzed for the hexocytidindiphosphoric acid barium salt.

*Analysis of the Brucine Salts.*

The most insoluble fraction of the brucine salt analyzed as follows:

0.1001 gm. of the substance on combustion gave 0.1999 gm. of  $\text{CO}_2$  and 0.0606 gm. of  $\text{H}_2\text{O}$ .

0.2000 gm. of the substance gave 11.4 cc. of nitrogen gas at  $T = 21^\circ\text{C}$ . and  $P = 751$  mm.

0.3000 gm. of the substance gave on fusion 0.288 gm. of  $\text{Mg}_2\text{P}_2\text{O}_7$ .  
 $\text{C}_{11}\text{H}_{13}\text{N}_2\text{P}_2\text{O}_{13}\text{C}_{92}\text{H}_{104}\text{N}_8\text{O}_{16}$     Calculated.    C 54.29, H 6.15, N 6.15, P 2.73.  
 +14 $\text{H}_2\text{O}$ .                                      Found.            C 54.45, H 6.77, N 6.54, P 2.67.

The most soluble fraction analyzed as follows:

0.1004 gm. of the substance gave on combustion 0.1990 gm. of  $\text{CO}_2$  and 0.0591 gm. of  $\text{H}_2\text{O}$ .

0.2000 gm. of the substance gave 12.0 cc. of nitrogen gas at  $T = 23^\circ\text{C}$ . and  $P = 752$  mm.

0.3000 gm. of the substance gave 0.0287 gm. of  $\text{Mg}_2\text{P}_2\text{O}_7$ .  
 $\text{C}_{10}\text{H}_{17}\text{N}_3\text{P}_2\text{O}_{12}\text{C}_{92}\text{H}_{104}\text{N}_8\text{O}_{16}$     Calculated.    C 54.10, H 6.64, N 6.81, P 2.75.  
 +14 $\text{H}_2\text{O}$ .                                      Found.            C 54.05, H 6.58, N 6.85, P 2.67.

This material was then converted into the barium salt. It must be remarked however, that the elementary compositions of the brucine salts of the above two nucleotides do not differ sufficiently one from another to permit identification of the nucleotide on the basis of the analysis of the brucine salts. Often, samples, which on the basis of the analysis of the brucine salt seemed to be the cytidin nucleotide, proved on conversion into the barium salt to be the thymidin nucleotide.

*Conversion of Brucine Salts into Barium Salts.*

For this purpose the brucine salts were dissolved in 35 per cent alcohol, an excess of ammonia water added, and the product allowed to stand in the refrigerator. The brucine was then removed by filtration, the filtrate was again concentrated and allowed to stand to permit further crystallization of brucine. The operation was repeated as long as brucine crystallized out. From the final solution the nucleotides were precipitated by a 25 per cent solution of neutral lead acetate. The lead salt was washed repeatedly with water, filtered, suspended in water, and

freed from lead by means of hydrogen sulfide. The filtrate from lead acetate is concentrated under diminished pressure at room temperature and then converted into the barium salt.

The hexothymidin salt was then converted into the crystalline form.

*Preparation of Crystalline Hexothymidindiphosphoric Barium Salt.*

9 gm. of the barium salt obtained from the brucine salt were taken up in 500 cc. of water and shaken for 1 hour. Part remained insoluble. All was allowed to stand over night when a crystalline deposit was found covering the undissolved amorphous material. The mixed deposit was then taken in 1.5 liters of water at 30°C. and shaken for 1 hour in a shaking machine. The insoluble part was removed by filtration and the filtrate concentrated under diminished pressure at room temperature to a volume of 350 cc. After several hours of standing there appeared a crystalline deposit consisting of long needles grouped into star-shaped aggregates.

The composition of the substance was the following:

0.1050 gm. of the substance gave 0.0700 gm. of  $\text{CO}_2$  and 0.0182 gm. of  $\text{H}_2\text{O}$ .

0.1719 gm. of the substance used for Kjeldahl nitrogen estimation required for neutralization 4.97 cc. of 0.1 N acid.

0.2579 gm. of the substance gave on fusion 0.0830 gm. of  $\text{Mg}_2\text{P}_2\text{O}_7$ .

$\text{C}_{10}\text{H}_{13}\text{N}_3\text{P}_2\text{O}_{12}\text{Ba}_2$ . Calculated. C 18.37, H 1.97, N 3.89, P 8.62.

Found. C 18.11, H 1.93, N 4.04, P 8.97.

*Barium Salt of Hexocytidindiphosphoric Acid.*

This was prepared from the brucine salt in the same manner as the thymidin salt. As yet it has not been converted into the crystalline form.

It analyzed as follows:

0.1052 gm. of the substance gave on combustion 0.0658 gm. of  $\text{CO}_2$  and 0.0260 gm. of  $\text{H}_2\text{O}$ .

0.1870 gm. of the substance employed for Kjeldahl nitrogen estimation required for neutralization 8.21 cc. of 0.1 N acid.

0.2805 gm. of the substance gave 0.0946 gm. of  $\text{Mg}_2\text{P}_2\text{O}_7$ .

$\text{C}_{10}\text{H}_{13}\text{N}_3\text{P}_2\text{O}_{12}\text{Ba}_2$ . Calculated. C 17.05, H 1.86, N 5.97, P 8.81.

Found. C 17.05, H 2.12, N 6.17, P 9.40.



## CREATININE AND CREATINE IN MUSCLE EXTRACTS.

### I. A COMPARISON OF THE PICRIC ACID AND THE TUNGSTIC ACID METHODS OF DEPROTEINIZATION.

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(Received for publication, June 29, 1921.)

Since the publication by Folin (1) in 1914 of the determination of creatinine and creatine in the filtrates from the deproteinization of blood, milk, and tissues by saturated aqueous picric acid solution plus the addition of solid picric acid, criticisms of the method have been made by Benedict (2), McCrudden and Sargent (3), Hunter and Campbell (4), and others. The most serious objection to the method is that when the picric acid filtrate is heated for the transformation of the creatine to creatinine, certain changes apparently take place which yield results that are higher than those obtained when filtrates from other methods of precipitation are similarly treated.

Although Folin and Wu (5) in their system of blood analysis, for obvious reasons of convenience, do not use the picric acid deproteinization, they, nevertheless, still feel that the original process can be utilized when the proper precautions are taken. Because of this controversy and because of the fact that the direct picric acid precipitation of tissue extracts, when creatinine and creatine alone are to be determined, has its elements of convenience in the reduction of dilution and manipulation, a comparison was made of this method and the method of deproteinization with sodium tungstate and  $2/3 \times$  sulfuric acid.

In this as in the studies to follow, the standards were made from purified creatinine zinc chloride prepared according to Benedict (6). The stock solution was standardized against  $0.5 \times$  potassium bichromate and the requisite dilutions made therefrom according to Folin (7). For each series of determinations adequate standard solutions were prepared so that there would be at hand

one with which the unknown solutions would closely correspond. This was considered necessary since the curves of Hunter and Campbell (8), while valid for the conditions under which they worked, may or may not be suitable for use in these studies. The picric acid was purified according to the method of Folin and Doisy (9) and satisfied the requirements stated in their report. Freshly saturated picric acid solutions were always used and were made up in the ratio of 2.5 gm. of the acid to every 100 cc. of distilled water. They were well shaken and the portions that were used for the reaction with the standards were always filtered clear through cotton.

The muscle extracts were prepared from fresh cleaned tissue obtained from the posterior limbs of albino rats killed by ether. The tissue was first ground in a meat chopper, then macerated with an equal weight of fine sand in a mortar, and mixed with an equal volume of Tyrode's solution and 5 cc. of toluene. The suspension was put into a small press and the expressed extract was measured and diluted with an equal volume of Tyrode's solution. 5 cc. portions were used in all the tests.

After several trials the following procedures were developed for the determination of the preformed and total creatinine in the muscle extracts.

In the picric acid deproteinization 5 cc. of the diluted extract are measured into a test-tube or centrifuge tube previously marked at the 15 cc. level, and 10 cc. of a saturated picric acid solution in distilled water are added. After the addition of a small amount of solid picric acid the whole is thoroughly shaken and centrifuged for 3 or 4 minutes. It was found that a better sedimentation is obtained if the contents of the tube are vigorously mixed with a small stirring rod immediately before centrifuging. 10 cc. of the filtrate, obtained by pouring the supernatant fluid in the tube through a bit of cotton in a funnel, are measured into a small flask or vial. 1 cc. of distilled water is added and 1 cc. of 20 per cent sodium hydroxide accurately measured. This ratio between the picric acid and the sodium hydroxide is that used by Folin and Wu (5). The standards for comparison are each made up of 1 cc. of stock creatinine solution containing the appropriate amount of creatinine (0.05 and 0.10 mg. per cc. for the extracts used in these studies), 10 cc. of saturated picric acid, and 1 cc. of

20 per cent sodium hydroxide. Both the unknown and the standard solutions are allowed to stand for 10 minutes and are then compared in the colorimeter. Sometimes it is necessary to filter off through cotton a light flocculent precipitate from the unknown solutions. The addition of the sodium hydroxide should be made with the same pipette and with the same procedure in all cases. It should be noted that in all circumstances the standard solutions are identical with the unknown solutions with respect to the amount of picric acid, and the amount of sodium hydroxide, and are closely similar in colorimetric value.

For the determination of the total creatinine, that is to say, the creatine as creatinine plus the preformed creatinine, 10 cc. of the filtrate from a second 5 cc. sample of extract precipitated as described are put in a small Erlenmeyer flask, diluted with 10 cc. of distilled water, and heated at the boiling point for 2 hours on an electric hot-plate. Partial evaporation is allowable. Complete evaporation is disastrous and is prevented by the addition of small amounts of water from time to time as the occasion demands. During the last half hour of heating the solution may be allowed to concentrate to about 3 or 5 cc. although a 10 cc. final volume does not affect the end-result. The flask is then removed from the hot-plate, cooled to room temperature, and the contents are made to about 16 cc. with distilled water. 1 cc. of 20 per cent sodium hydroxide is added and the mixture is allowed to stand for 10 minutes when it is transferred to a 100 cc. flask and diluted to the mark. The solution so made up is compared with the appropriate standard. It has been found that for extracts prepared as described a standard consisting of 1.5 mg. of creatinine plus 10 cc. of saturated picric acid solution and 1 cc. of 20 per cent sodium hydroxide diluted to 50 cc. after 10 minutes standing is satisfactory.

The final procedure developed for the determination of creatinine and creatine in the tungstic acid deproteinization method is as follows. 5 cc. of the diluted muscle extract are put into a test-tube or centrifuge tube marked at the 15 cc. level, and 5 cc. of distilled water are added. Then 2 cc. each of a 10 per cent solution of sodium tungstate and  $\frac{2}{3}$  N sulfuric acid are added, the whole is made to 15 cc. with water, shaken thoroughly, and centrifuged. For the creatinine determination the supernatant solution

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is filtered through a bit of cotton and 10 cc. of the filtrate are transferred to a small vial. 10 cc. of saturated picric acid are added and 1 cc. of 20 per cent sodium hydroxide. The standards for these unknown solutions are made by taking 1 cc. of the appropriate original creatinine concentrations, adding 10 cc. saturated picric acid, 9 cc. of distilled water, and 1 cc. of sodium hydroxide. Both standard solutions and unknown solutions are allowed to stand for 10 minutes and are then compared in the colorimeter as previously described. For the determination of the total creatinine, 10 cc. of the filtrate from the tungstic acid precipitation are put into a small Erlenmeyer flask, diluted with 10 cc. of distilled

TABLE I.

*The Amounts of Preformed and Total Creatinine in Muscle Extracts after Deproteinization by Picric Acid and Tungstic Acid.*

Preformed creatinine.		Total creatinine.	
Method...	Picric acid.	Picric acid.	Tungstic acid.
<i>mg.</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>
0.125	0.125	7.32	7.32
0.288	0.300	9.89	10.25
0.143	0.112	5.14	4.95
0.120	0.117	5.49	5.40
0.108	0.114	5.49	5.54
0.111	0.114	5.45	5.40
0.071	0.073	5.22	5.22

water, 1 cc. of  $N$  hydrochloric acid is added, and the whole is heated for 2 hours as described for the picric acid filtrates, save that in this case the final solution should not exceed 2 or 3 cc. in volume. The flask is removed from the hot-plate, cooled, and 10 cc. of picric acid solution are added and 1 cc. of 20 per cent sodium hydroxide. The mixture is allowed to stand for 10 minutes, is then transferred to a 100 cc. graduated flask, and diluted to the mark. The standard for this determination is exactly the same as that for the analysis of the picric acid filtrate.

When the methods as outlined are carried out on one and the same extract concordant results are obtained as shown in Table I. Parallel determinations were made in all cases.



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## CREATININE AND CREATINE IN MUSCLE EXTRACTS.

### II. THE INFLUENCE OF THE REACTION OF THE MEDIUM ON THE CREATININE-CREATINE BALANCE IN INCUBATED EXTRACTS OF MUSCLE TISSUE OF THE ALBINO RAT.

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(Received for publication, June 29, 1921.)

The object of this investigation was the determination of the changes that take place in the creatinine and creatine content of extracts of muscle tissue of the albino rat when incubated at body temperature for 24 hours when the reaction of the extract is buffered to neutrality or alkalinity, and when the extract is unbuffered save by the Tyrode's solution used as diluent, and is allowed to develop its own reaction which is slightly acid to rosolic acid. The study is to serve as a foundation for an inquiry into the factors concerned in the creatinine-creatine balance in such tissue extracts with the hope that some light may be thrown on the problems of the metabolism of these compounds.

There are those who have doubted that the demonstration by Stangassinger (1), Gottlieb and Stangassinger (2, 3), Rothmann (4), Mellanby (5), and Myers and Fine (6) of an increase in creatinine accompanied by a decrease in creatine content of muscle tissue or muscle extracts in *in vitro* experiments is a valid indication that such a process occurs in the living organism. The objections raised to such an application of the findings have been based on evidence which has its contradictory phases, while on the other hand, the main facts of the strictly laboratory tests consistently point in one direction. The uniformity of the results of these latter methods of attack can be attributed to the elimination of the interfering factors of digestion, assimilation, utilization, bacterial action, and the probable influence of organs other than the muscles on the reaction being studied. It is not necessary to go into the literature dealing with

this controversy, for to those interested in the problem its main features are well known.

The results of the studies of autolyzed muscle tissue or extracts have shown that the increase of the creatinine content in such preparations occurs whether the reaction of the medium is acid, neutral, or alkaline. But the data are conflicting with respect to the relative influence of the reaction on the amount of creatine formation. The recent report of Hahn and Barkan (7) on the effects of sodium hydroxide and hydrochloric acid on this change in aqueous solutions of creatine while of interest is hardly directly comparable with the studies in which tissue extracts were used.

For the purposes of this study extracts were prepared from the voluntary muscles of the hind limbs of albino rats as described in the preceding paper (8). Rats of the same sex and age were used within each series, although the sex and age differed for the different series. For each series a set of sixteen centrifuge tubes was used and into each tube 5 cc. of muscle extract were measured, using the same pipette throughout. To the first group of four tubes there were added 4 drops of distilled water; to the second group 4 drops of Henderson's (9) phosphate mixture; and to the third 4 drops of a saturated solution of  $\text{Na}_2\text{HPO}_4$ . The remaining four lots were used for the estimation of the preformed and total creatinine of the fresh extract. 0.5 cc. of toluene was added to the above mixtures and they were thoroughly mixed by means of a fine stream of air blown through a glass capillary dipped to the bottom of the tubes. When the reaction of the various groups was tested with rosolic acid, they were found to be slightly acid, neutral, and alkaline, respectively, both before and after incubation. After the contents of the tubes had been prepared as described they were incubated for 24 hours usually at a temperature of  $38^\circ$ , although some lots were kept at  $36^\circ$  and  $40^\circ$ . After incubation the preformed and total creatinine were determined according to the picric acid deproteinization method previously described (8). Parallel determinations were made and the reported values represent their averages. The statistical values of the parallel determinations are given in Table I in terms of 0.1 mm. and demonstrate that a considerable degree of reliance can be placed upon the findings.

Turning now to a consideration of the results of these experiments the figures in Table II are given. They represent in mg. the amounts of total and preformed creatinine found in the fresh extracts and in the extracts after incubation under the conditions described. The percentage increase is also tabulated as are the statistical values for the series as a whole. No figures are given for creatine since they are obtained by difference and calculation and would add nothing to the argument.

It is evident that here, as with other workers, there has been an increase in the creatinine content of the extracts on incubation

TABLE I.

*Statistical Values of the Colorimetric Readings of the Parallel Determinations.*

	Mean difference.	Probable error of mean.	Standard deviation.	Probable error of standard deviation.
	0.1 mm.	0.1 mm.	0.1 mm.	0.1 mm.
Creatinine before incubation .....	2.2	0.25	1.76	0.18
Creatinine after incubation.				
Acid .....	2.4	0.31	1.98	0.22
Neutral .....	2.4	0.31	1.84	0.22
Alkaline .....	3.0	0.38	2.24	0.27
Creatinine; all determinations .....	2.5	0.15	1.97	0.11
Total creatinine before incubation.	2.1	0.25	1.80	0.18
Total creatinine after incubation.				
Acid .....	2.2	0.39	2.03	0.28
Neutral .....	3.6	0.49	1.94	0.35
Alkaline .....	2.6	0.46	1.91	0.32
Total creatinine; all determinations.	2.4	0.18	1.94	0.13

whether the reaction of the medium was acid, neutral, or alkaline. This increase is statistically valid as measured by the usual criterion that the probable error of the mean must be contained in the difference between the means at least twice, and three times for definitely satisfactory differences. No changes in the amounts of total creatinine occur on incubation when the same standard of validity is applied. Such being the case and since there has been neither gain nor loss of total creatinine under these conditions, the increase in the creatinine must perforce have been at the expense of the creatine. Since the muscle extracts exhibiting this phenomenon were extracts made with

TABLE II  
*Changes in the Creatinine-Creatine Balance on Incubation.*

	Preformed creatinine.						Total creatinine.						
	Fresh.	Incubated.						Fresh.	Incubated.				
		Acid.		Neutral.		Alkaline.			Acid.	Neutral.		Alkaline.	
		mg.	per cent increase	mg.	per cent increase	mg.	per cent increase			mg.	per cent increase		mg.
Mean.....	0.055	0.134	143.6	0.199	261.8	0.166	5.19	5.20	0.2	5.20	0.2	5.22	0.6
Standard deviation.	0.064	0.167	161.0	0.228	256.2	0.184	5.41	5.43	0.4	5.43	0.4	5.43	0.4
Probable error of mean.....	0.067	0.074	10.4	0.094	40.3	0.077	3.33	3.37	1.2	3.33	0.0	3.33	0.0
Probable error of standard deviation.....	0.073	0.145	98.6	0.211	189.0	0.163	5.07	4.85	-4.3	4.98	-1.8	5.05	-0.4
	0.074	0.150	102.7	0.188	154.1	0.167	4.42	4.42	0.0	4.52	2.3	4.42	0.0
	0.074	0.171	131.1	0.286	286.5	0.214	5.55	5.49	-1.1	5.46	-1.6	5.48	-1.2
	0.078	0.148	89.7	0.185	137.2	0.150	4.44	4.42	-0.5	4.50	1.4	4.45	-0.4
	0.080	0.135	68.7	0.160	100.0	0.152	4.83	4.64	-3.9	4.64	-3.9	4.62	-4.4
	0.081	0.195	140.7	0.286	253.1	0.212	5.53	5.53	0.0	5.53	0.0	5.49	-0.7
	0.081	0.213	163.0	0.301	271.6	0.230	5.24	4.79	-8.6	5.28	0.8	5.29	1.0
	0.088	0.137	55.7	0.166	88.6	0.157	5.06	4.99	-1.3	4.93	-2.6	4.93	-2.6
	0.116	0.158	36.2	0.188	62.1	0.162	4.83	4.80	-0.6	4.83	0.0	4.80	-0.6
Mean.....	0.077	0.152	100.0	0.208	175.0	0.169	4.91	4.83	-1.5	4.88	-0.4	4.88	-0.6
Standard deviation.	0.014	0.011	47.8	0.018	85.6	0.012	0.60	0.57	2.6	0.58	1.7	0.59	1.4
Probable error of mean.....	0.003	0.002	9.1	0.004	16.7	0.002	0.12	0.11	0.5	0.11	0.3	0.12	0.3
Probable error of standard deviation.....	0.002	0.002	6.6	0.003	11.8	0.002	0.08	0.08	0.4	0.08	0.2	0.08	0.2

Tyrode's solution which simulates to a considerable degree the medium in which the reactions of the living organism take place, I am of the opinion that we are justified in assuming, until it has been disproved by critical experiment, that there occurs in the muscles of the living organism a formation of creatinine from the muscle creatine and that the endogenous source of the urinary creatinine is the muscle creatine.

This lack of destruction of total creatinine just discussed confirms the findings of Mellanby (5) and Myers and Fine (6) and fails to substantiate the results of Gottlieb and Stangassinger (2). Experiments where putrefaction was allowed to occur, and which will be presented presently, tell another story.

Now when the percentage increase in creatinine is considered it is seen that this increase is regulated in part by the reaction of the medium, for it is least in the acid solutions, greatest in the neutral solutions, and between the two in the alkaline solutions. This relationship is consistently constant in all of the twelve experiments reported and is substantiated by the statistical calculations. It is not in agreement with the result of Rothmann (4), Myers and Fine (6), or Hahn and Barkan (7). For the two former found an apparent acceleration of the reaction by acid and the latter that alkali retarded the change of creatinine to creatine as compared with acid. The studies of Hahn and Barkan (7), however, are hardly comparable with the studies made with tissue extracts. When one looks at the results of Myers and Fine (6) given in Table VII of their paper, it is seen that when the autolyzing mixtures were buffered to neutrality by phosphate mixture a somewhat greater creatinine formation took place than when the tissue was treated with water alone. My results confirm this finding in principle. Nevertheless the studies of Myers and Fine (6) are not strictly comparable with mine inasmuch as they used whole muscle tissue, their periods of autolysis were extended over a longer period and they used an acid not normally found in muscle tissue.

Such being the case it is evident that a slightly acid or an alkaline reaction retards the transformation of creatine to creatinine in muscle extracts when incubated for 24 hours at body temperature. This transformation occurs at a maximum when the reaction of the digesting mixtures is buffered to neutrality by a

phosphate mixture. These facts serve as a partial explanation of the observations of Underhill (10) that creatinuria is frequently an accompaniment of induced acidosis and of Underhill and Baumann (11) that a marked increased creatine excretion may be found in experimental alkalosis, and other apparently anomalous results of the studies of creatinuria, if we admit that the urinary creatinine is largely derived from the muscle creatine, and in spite of the opinions of Denis and Minot (12) and Gamble and Goldschmidt (13) that the acid-base equilibrium has nothing to do with the condition. For since it is shown that both a slight acidity and an alkalinity retard the transformation of creatine to creatinine in muscle extracts it is possible to consider that if similar tendencies are present in the living organism, even though fleeting, they may give rise to similar effects, and if that phase of muscle metabolism which results in creatine formation continues at the same or even a diminished rate, there is produced a relatively greater concentration of creatine in the circulation, part of which at least finds its way to the kidneys and is excreted. That such an increase in blood creatine can occur and continue for days under changed conditions of muscular activity I have already demonstrated (14), though this phase of the problem is not necessarily at present connected with the question of creatinuria and acid-base equilibrium.

Since these experiments demonstrate conclusively that there is no loss of total creatinine on incubation under sterile conditions the figures given in Table III are particularly interesting, from the point of view of the contention of Gottlieb and Stangassinger (3) of the presence in muscle tissue of creatinine—and creatine—destroying enzymes. The results in this table were obtained from extracts which had been allowed to undergo putrefaction during the incubation. It will be seen that there has occurred a marked loss of total creatinine that is statistically valid. This supports the findings of Mellanby (5) that only when bacterial decomposition occurs does there take place a destruction of creatine or creatinine, and plainly shows the cause of the results reported by the proponents of the "creatinase" and "creatase" theory. However, it is quite probable that the transformation of creatine to creatinine in muscle extracts is brought about by an enzyme, in view of the fact that the change occurs in the



TABLE III.  
*Effect of Putrefaction on Changes in Creatinine-Creatine Balance During Incubation.*

Preformed creatinine.										Total creatinine.			
	Fresh.			Incubated.				Fresh.	Incubated.				
	Acid.		Neutral.	Alkaline.		Acid.	Neutral.		Alkaline.				
	mg.	per cent increase		mg.	per cent increase					mg.	per cent increase		
Mean .....	0.069	0.500	624.9	0.540	682.8	0.509	637.7	5.55	1.99	3.00	45.9	1.28	76.9
Standard deviation.	0.104	0.544	422.9	0.588	465.5	0.653	527.9	4.48	0.57	5.36	0.0	0.83	81.5
Probable error of mean .....	0.118	0.300	154.2	0.273	131.4	0.632	435.5	5.36	2.15	1.18	77.2	2.05	50.6
Probable error of standard deviation .....	0.121	0.588	386.0	0.741	512.3	0.690	470.1	5.30	1.12	1.18	77.2	0.96	81.9
Mean .....	0.103	0.483	396.0	0.535	448.0	0.621	517.8	5.17	1.46	3.18	40.8	1.43	72.7
Standard deviation.	0.021	0.111	167.2	0.169	200.1	0.068	76.7	0.41	0.65	1.71	31.4	0.72	16.7
Probable error of mean .....	0.007	0.037	56.4	0.057	67.5	0.023	26.0	0.14	0.21	0.58	10.6	0.24	5.7
Probable error of standard deviation .....	0.005	0.026	39.8	0.040	47.6	0.016	18.3	0.10	0.16	0.41	7.5	0.17	4.0

neutral buffered solutions at a much greater rate than in either the acid or alkaline solutions of muscle extracts or aqueous solutions, or in aqueous solutions uncatalyzed by acid or alkali.

It is interesting to note that the increase of creatinine in these putrefying extracts is much greater than is that which occurs in sterile mixtures. Whether this is a true increase or whether other products are produced by the bacterial action which give the color test for creatinine I am unable to state. The greatest increase takes place in these solutions whose reaction is alkaline. It is also interesting to note that the destruction of the total creatinine occurs about equally well in acid or alkaline media while it is much less in the solutions buffered to neutrality. This may be a direct destruction of creatine or the creatine may first be changed to creatinine which is then destroyed. An explanation of these phenomena is beyond the scope of the present paper, however. The main fact to be gathered is that the only time a destruction of total creatinine is demonstrable is when putrefaction occurs in the incubating extracts.

It should be noted in conclusion that slight differences in temperature during incubation result in differences in amounts of creatinine formation, in that at the lower temperatures the transformation was less. This confirms Myers and Fine (6).

#### SUMMARY AND CONCLUSIONS.

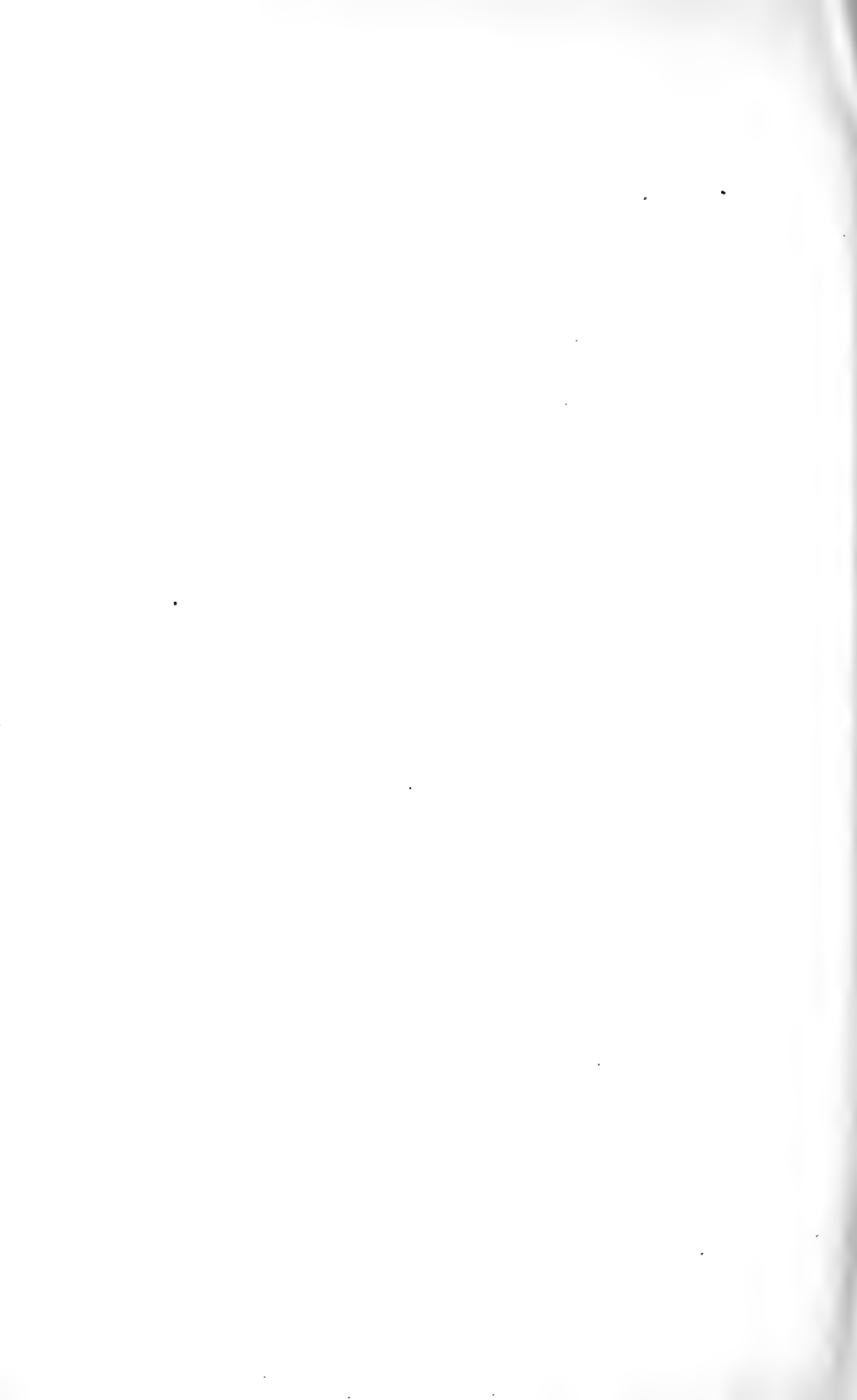
When extracts of muscle tissue of the albino rat are incubated at body temperature for 24 hours there occurs an increase in the creatinine content the relative degree of which depends in part upon the reaction of the incubated extract. When the extract is allowed to develop its own reaction, which is slightly acid to rosolic acid, an increase of 100 per cent takes place. When the extract is buffered to neutrality by phosphate mixture the increase is 175 per cent, and when the extract is made slightly alkaline the increase is 124 per cent. Since there is no change in the total creatinine content of these extracts this increase in creatinine must take place at the expense of the creatine present. Moreover, since the conditions of the experiments simulate to a considerable degree conditions in the living tissue in that the reactions took place in muscle extract diluted with Tyrode's solution it is probable that creatinine is formed from creatine in muscle tissue in the living

organism. The apparent anomaly of an increased creatine excretion in conditions of experimental acidosis and alkalosis is explicable in part on the basis of the retardation of creatinine formation from creatine in incubated muscle extracts when the reaction is slightly acid or alkaline. If similar effects are produced in the organism the continued production of creatine as a result of a phase of muscle metabolism would result in a relatively greater concentration of this in the blood and its excretion in larger amounts in the urine.

It is probable that the transformation of creatine to creatinine in the muscle extracts is facilitated by an enzyme, but no evidence is afforded of the presence in such extracts of any "creatinase" or "creatase." The only time when a destruction of creatinine or creatine takes place is when the extracts undergo putrefaction.

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## STUDIES OF THE THYROID APPARATUS.

### IV. THE INFLUENCE OF PARATHYROID AND THYROID TISSUE ON THE CREATININE-CREATINE BALANCE IN INCUBATED EXTRACTS OF MUSCLE TISSUE OF THE ALBINO RAT.

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(Received for publication, June 29, 1921.)

The accumulated results of many investigators lead to the idea of a possible relation between parathyroid function, muscle tonus, and creatine-creatinine metabolism.

The occurrence of tetany after removal of the parathyroids and the fact that animals in a state of high neuromuscular tension are more apt to die from acute tetania parathyreopriva after parathyroidectomy than are animals of low tension (1), indicate a connection between parathyroid function and muscle tone. The latter is obviously related to the degree of the activity of the nervous system. The factors concerned in this have been discussed in another place (2).

It is not the purpose of this paper to analyze the controversy concerning the relation of muscle creatine to urinary creatinine which developed from the theory of Folin (3) that urinary creatinine represents a special phase of protein metabolism, probably that of the muscles. It was pointed out in the preceding paper (4) that the present available evidence supports the hypothesis that the source of the urinary creatinine is the muscle creatine. Until such a conception is disproved we shall use it as a working basis for experimentation and interpretation.

The studies on the place of creatine in muscle metabolism which are of interest for our present purpose are those of Weber (5), Cathcart, Henderson, and Paton (6), and Hammett (7). They all indicate that creatine is a by-product of that phase of muscle metabolism concerned in muscle tone. The recent report of Stearns and Lewis (8) on creatinuria in women suggest a relation

between nervous strain with its consequent high neuromuscular tension, and an increased creatine excretion. The relation between muscle tone and creatine-creatinine metabolism is brought out by these observations.

Underhill and Saiki (9), Burns (10), and Greenwald (11) have shown that an increased creatine excretion occurs after the parathyroids have been removed from the experimental animals, and Henderson (12) found an increase in the creatine content of the muscles of parathyroidectomized dogs. This demonstrates a relation between creatine-creatinine metabolism and the parathyroids. This relation may be direct or indirect though the results of these experiments tend to show a direct influence.

This brief outline of the evidence on which the present investigation was begun will suffice as a general statement of the problem. Its points of contact are many. At one point it touches the influence of behavior, in the sense of Paton (13), on metabolism; a problem the importance of which can hardly be over-emphasized.

In view of this relation of parathyroid function, muscle tonus, and creatine-creatinine metabolism it seemed worth while to determine the effect of parathyroid tissue on the changes induced in the creatine-creatinine balance of muscle extracts by incubation for 24 hours at body temperature. In these experiments muscle extracts were made as previously described from muscle tissue of albino rats, and the preformed and total creatinine determinations on the incubated extracts and the fresh material were carried out according to the picric acid deproteinization method discussed in a preceding paper (4). Rats of the same age and sex were used for each individual series but different ages and sexes were used for the different series.

A set of forty centrifuge tubes was used for each series. In all of them there were first put 4 drops of Tyrode's solution. Into each of twelve of them there were then placed three parathyroid glands removed from albino rats immediately after death by ether. Into each of another lot of twelve tubes there were placed three bits of thyroid tissue, each of approximately the same size as a parathyroid gland. Each tube in the thyroid set was comparable to a tube in the parathyroid set since it contained thyroid tissue from the same lobes of the same rats from which the para-

thyroids were taken for its mate. This was done not only for the purpose of exact control, since some thyroid tissue occasionally contaminated the removed parathyroids, but also because it was desired to determine the effect of thyroid tissue on the changes induced by incubation. To the third set of twelve tubes no tissue was added. The remaining four tubes were reserved for use in the determination of the preformed and total creatinine in the fresh extract. There were now added to each of the forty tubes 5 cc. of muscle extract made from the tissues of the same rats from which the parathyroids had been taken.

Since it had been found that the reaction of the incubating extract has a marked influence on the degree of formation of creatinine (4), and since we had no information as to the effect of the reaction on the possible activity of the parathyroids in experiments of this type, it was thought advisable to study the parathyroid and thyroid effects in extracts allowed to develop their own reaction, which is slightly acid to rosolic acid; in extracts buffered to neutrality with Henderson's (14) neutral phosphate mixture; and in extracts made alkaline with a few drops of a saturated solution of  $\text{Na}_2\text{HPO}_4$ . Consequently, there were added to the first four tubes of each of the three sets of twelve, 4 drops of distilled water; to the second four tubes 4 drops of the phosphate mixture; and to the third four tubes 4 drops of the sodium phosphate solution, just as was done in the small series reported in the preceding paper (4). In this way there was obtained a series of rigidly controlled experiments which would show the effects, if any, of the parathyroid and thyroid tissue on the changes in the preformed and total creatinine of muscle extracts in acid, neutral, and alkaline solutions, on incubation for 24 hours at body temperature. Parallel determinations were made throughout. The variability of the parallel determinations was but a little above that found for the experiments made without the addition of parathyroid and thyroid tissue and is consequently not recorded.

The results of these procedures are given in Tables I to IV. In Table I there are recorded the amounts of preformed creatinine in the fresh extract and in the incubated extract with and without the addition of parathyroid tissue in slightly acid, neutral, and alkaline media. The percentage increase in creatinine and the

difference in the percentage increase of the extracts containing the parathyroids from the increase that took place in the controls are also given. Below these values there have been recorded those statistical calculations essential for a determination of the validity of the differences. In Table II the same data are given for the extracts incubated in the presence of thyroid tissue. In Tables III and IV the same data are recorded for the total creatinine with and without the addition of parathyroid and thyroid tissue, respectively.

An inspection of Tables I and II shows that the increased creatinine formation occurs in the controls, in the presence of parathyroid tissue, and in the presence of thyroid tissue; that it is least when the extract is allowed to develop its own reaction, is greatest where the reaction has been buffered to neutrality, and is between the two when an alkaline reaction is maintained. This is a confirmation of the results presented in the preceding paper (4).

From Table I it is evident that the addition of parathyroid tissue to muscle extracts has resulted in a partial inhibition of the formation of creatinine. This retardation occurs in acid and neutral reactions. It occurs to about the same degree in the acid and alkaline extracts, and to a greater degree in the neutral. These differences are statistically valid. From the fact that the maximum tendency to creatinine formation takes place in the extracts buffered to neutrality and the fact that the maximum tendency of the parathyroids to retard this process takes place in solutions of the same reaction, we have almost conclusive evidence of a participation of these glands in creatine-creatinine metabolism. This is further strengthened by the observation that in those extracts to which thyroid tissue was added no such retardation is found that is valid, as can be seen by an inspection of Table II. It is a parathyroid effect, pure and simple. Nor is there any evidence that the addition of the thyroid tissue has any influence whatever on the transformation of creatine to creatinine in muscle extracts.

Turning now to a consideration of the changes induced in the total creatinine by incubation in the presence of parathyroid and thyroid tissue, it is seen from Tables III and IV that while we get a hint of a possible decrease in total creatinine in the acid





TABLE II.  
*The Influence of Thyroid Tissue on the Changes in the Performed Creatinine of Muscle Extracts on Incubation.*

Incubated.			Acid.				Neutral.				Alkaline.						
Series.	Fresh.		Thyroid.		Controls.		Difference.	Thyroid.		Controls.		Difference.	Thyroid.		Controls.		Difference.
	mg.	per cent in-crease	mg.	per cent in-crease	mg.	per cent in-crease		mg.	per cent in-crease	mg.	per cent in-crease		mg.	per cent in-crease			
XVII	0.081	0.175	116.00	140.7	140.7	24.70	-24.70	0.258	218.50	0.286	253.1	-34.60	0.198	144.40	0.212	161.7	-17.3
XV	0.055	0.144	162.00	134	143.6	18.40	0.208	278.20	0.199	261.8	16.40	0.165	200.00	0.166	201.8	-1.8	
XIV	0.064	0.145	126.60	167	161.0	-34.40	0.221	245.30	0.228	256.2	-10.90	0.173	170.30	0.184	187.5	-17.2	
XIII	0.074	0.168	127.00	171	131.1	-4.10	0.268	262.20	0.286	286.5	-24.30	0.201	172.70	0.214	189.2	-16.5	
XII	0.073	0.150	105.50	145	98.6	6.90	0.212	190.40	0.211	189.0	1.40	0.158	116.40	0.163	123.3	-6.9	
XI	0.081	0.215	165.40	213	163.0	2.40	0.296	265.40	0.301	271.6	-6.20	0.226	174.00	0.230	184.0	-5.0	
X	0.088	0.127	44.30	137	55.7	-11.40	0.183	108.00	0.166	88.6	19.40	0.143	62.50	0.157	78.4	-15.9	
Mean.....	0.074	0.161	121.00	166	127.7	-6.70	0.235	224.00	0.240	229.5	-5.50	0.181	149.30	0.189	160.8	-11.5	
Standard deviation.....	0.010	0.027	37.50	0.028	35.5	17.00	0.037	53.10	0.047	64.2	18.40	0.027	43.40	0.027	41.3	6.2	
Probable error of mean.	0.003	0.007	9.60	0.007	9.0	4.30	0.009	13.50	0.012	16.3	4.70	0.007	11.10	0.007	10.5	2.0	
Probable error of standard deviation....	0.002	0.005	6.70	0.005	6.4	3.00	0.007	9.50	0.008	13.3	3.30	0.005	7.80	0.005	7.4	1.1	

TABLE III.  
*The Changes in the Total Creatinine Content of Muscle Extracts Incubated with Parathyroid Tissue.*

Incubated.			Acid.				Neutral.				Alkaline.						
Series.	Fresh.		Parathyroid.		Controls.		Difference.	Parathyroid.		Controls.		Difference.	Parathyroid.		Controls.		Difference.
	mg.		mg.	per cent increase	mg.	per cent increase		mg.	per cent increase	mg.	per cent increase		mg.	per cent increase			
XVII	5.53	5.55	0.4	5.53	0.0	0.4	5.57	0.8	5.53	0.0	0.8	5.74	3.8	5.49	-0.7	4.5	
	5.19	5.23	0.8	5.20	0.2	0.6	5.23	0.8	5.20	0.2	0.6	5.23	0.8	5.22	0.6	0.2	
	5.41	5.49	1.5	5.43	0.4	1.1	5.31	-1.9	5.43	0.4	-2.3	5.49	1.5	5.43	0.4	1.1	
	5.55	5.29	-4.7	5.49	-1.1	-3.6	5.45	-1.8	5.46	-1.6	-0.2	4.82	-13.2	5.48	-1.3	-11.9	
	5.07	4.90	-3.4	4.85	-4.4	1.0	5.08	-0.2	4.98	-1.8	1.6	5.11	0.8	5.05	-0.4	1.2	
	5.24	4.74	-9.5	4.79	-8.6	-0.9	5.60	6.9	5.28	0.8	6.1	5.33	1.7	5.29	1.0	0.7	
X	5.06	4.65	-8.1	4.99	-1.4	-6.7	4.76	-5.9	4.93	-2.6	-3.3	4.98	-1.6	4.93	-2.6	1.0	
Mean.....	5.29	5.12	-3.3	5.18	-2.1	-1.2	5.29	-0.2	5.26	-0.7	0.5	5.24	-0.9	5.27	-0.4	-0.5	
Standard deviation...	0.18	0.33	4.1	0.29	3.0	2.7	0.27	3.6	0.22	1.2	2.8	0.29	5.2	0.20	1.2	4.8	
Probable error of mean.	0.05	0.08	1.0	0.07	0.8	0.7	0.07	0.9	0.06	0.3	0.7	0.07	1.3	0.05	0.3	1.2	
Probable error of standard deviation..	0.03	0.06	0.7	0.05	0.5	0.5	0.05	0.6	0.04	0.2	0.5	0.05	0.9	0.03	0.2	0.8	



extracts, yet the decreases are too insignificant to justify any conclusion. As a whole the figures make it quite evident that it is impossible to believe that either parathyroid or thyroid tissue have any power to change the total creatinine content of muscle extract treated as described.

These observations fail to substantiate the findings of Rowe (15) that when parathyroid (?) tissue is added to solutions of creatine a decrease of some 30 per cent or more is found to occur. Since Rowe used sheep's thyroid as the source of his destructive agent in the belief that considerable parathyroid tissue is distributed throughout the gland his experiments are really more comparable with those of Table IV, and which fail to show any valid evidence of a loss of total creatinine. In view of his methods of procedure and the great contamination of his material with thyroid tissue, his results can hardly have much bearing on the problem of parathyroid function and creatine-creatinine metabolism.

The fact that an increased creatine excretion and an increased creatine content of muscle tissue follow the loss of the parathyroid secretion is not of itself sufficient evidence that the parathyroids are concerned in creatine metabolism. But when these facts are correlated with the findings presented in this paper that the addition of parathyroid tissue to muscle extracts actually retards the transformation of creatine to creatinine during incubation, we are justified in concluding that the parathyroids are directly concerned in creatine metabolism. The exact rôle of these glands, or the phase of creatine metabolism in which the parathyroids exert their main activity is not as yet clear. Speculation would be premature, particularly in view of the fact of the increased creatine production in the organism when the parathyroids have been removed. The clarification of these points awaits further experimentation.

#### SUMMARY AND CONCLUSIONS.

Evidence is presented which demonstrates that the addition of parathyroid tissue to extracts of muscle tissue of albino rats retards the increase of creatinine formation normally taking place during incubation. This occurs in acid, neutral, or alkaline mixtures. The addition of thyroid tissue to similar extracts has no effect

upon the creatinine formation that is demonstrable by the methods used. Since the maximum retardation effect of the parathyroids occurs in solutions buffered to neutrality, while the maximum creatinine formation takes place at the same reaction, the conclusion is justified that this parathyroid effect is an expression of a direct influence of the parathyroids on creatine metabolism. This conclusion is supported by correlated observations reported in the text.

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## STUDIES OF ACIDOSIS.

### XVII. THE NORMAL AND ABNORMAL VARIATIONS IN THE ACID-BASE BALANCE OF THE BLOOD.

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(Received for publication, July 22, 1921.)

The possible variations in the acid-base balance of the blood may be stated as follows: the blood bicarbonate may be high, low, or normal, and in each of these conditions the pH may be high, low, or normal. There are as thus classified nine theoretically possible conditions. Only one of them is normal, that in which both bicarbonate and pH are within the normal limits. At the time of the first paper of this series (Van Slyke and Cullen, 1917) only two of the abnormal possibilities had come under clinical observation, that in which bicarbonate is low, and pH normal (compensated acidosis), and that in which bicarbonate is very low, and pH also low (uncompensated acidosis). Now, however, as the result of the recent work of Y. Henderson and Haggard, of Scott, of Milroy, of Collip, of Davies, Haldane, and Kennaway, of Grant and Goldman, of Peters and Barr, and of others, it is known that the other six abnormal possibilities can be produced experimentally, and that at least some of them occur clinically. For this reason it has seemed desirable to enlarge the view presented in our former paper in order to include within it these conditions.

*The Normal and Abnormal Ranges of Hydrion Concentration of the Blood Plasma and Other Extracellular Fluids.*—The average normal hydrion concentration of the blood plasma lies at or near the slightly alkaline point  $H^+ = 4 \times 10^{-8}$ , or  $pH = 7.4$ . This figure was estimated on the basis of material then available by L. J. Henderson in 1909, and has been confirmed by Lunds-gaard (1912), Hasselbalch, and other subsequent investigators, utilizing the gas chain method. Parsons (1917) working with

especial precautions showed that the actual pH value determined is that of the plasma, and that the pH of venous blood in a given individual is normally only 0.02 below that of arterial blood. The maximum normal range of variation of blood reaction in different individuals appears to be indicated by pH 7.30 to 7.50. It is possible that when errors of technique are more completely excluded this range will become still narrower. For a given individual in the resting condition the data of Parsons and of Hasselbalch indicate that the pH variation may be only a few units in the second decimal place.

Under extreme abnormal conditions the pH may fall as low as 6.95, but before this point is reached it appears that coma occurs, and, from the fact that lower pH values have not been observed, it is doubtful that further decrease is compatible with life. This was the lowest point observed by Hasselbalch and Lundsgaard (1912) in rabbits killed by prolonged breathing of air which contained  $\text{CO}_2$ . It was also the lowest point observed by Van Slyke, Austin, and Cullen (1920) in experiments on etherized dogs. A pH of 6.95 was in one instance determined electrometrically by Cullen (unpublished) in the blood of a nephritic man in coma a few hours before death.

By voluntary deep breathing, on the other hand, carbonic acid may be blown off until the blood alkalinity rises to a pH of 7.7 or 7.8 (Davies, Haldane, and Kennaway, 1920; Collip and Backus, 1920), at which point, however, symptoms of tetany appear (Grant and Goldman, 1920). It therefore appears that the extreme range of reaction compatible with life lies approximately between pH 7.0 and 7.8, and that the normal range is within limits no greater than pH 7.3 to 7.5, and possibly somewhat narrower.

Concerning the pH of the body fluids other than blood plasma our knowledge is limited, but such as it is indicates that these fluids approximate the blood plasma closely in their reaction. (By body fluids are meant the fluids within the body proper; such are lymph, cerebrospinal fluid, transudates, exudates, but not secretions such as gastric juice or urine.) Parsons and Shearer (1920) found in the cerebrospinal fluid a pH normal for blood plasma. Cullen and Boots in this Hospital (unpublished data) have observed a similar pH in joint fluids. In other body



fluids, data of Collip and Backus (1920) and of others have shown that the bicarbonate is normal for blood plasma. As there is reason to believe that the  $\text{CO}_2$  tension in these fluids approximates that of the arterial blood (Haggard and Henderson, 1919) it appears that a bicarbonate concentration normal for blood plasma indicates also a  $[\text{BHCO}_3]:[\text{H}_2\text{CO}_3]$  ratio, and therefore a pH in these fluids normal for blood plasma (B is used to indicate any monovalent base, such as Na or K,  $[\text{BHCO}_3]$  to indicate the bicarbonate concentration, and  $[\text{H}_2\text{CO}_3]$  the concentration of free carbonic acid).

*The Normal and Abnormal Ranges of the Blood Bicarbonate Concentration.*—Peters and Barr (1921) have reviewed the data in the literature and concluded therefrom that in normal men the total  $\text{CO}_2$  content of the whole blood under 40 mm.  $\text{CO}_2$  tension at  $38^\circ$  falls between 43 and 55 volumes per cent. The  $\text{CO}_2$  content of the plasma is about 8 volumes per cent higher (Joffe and Poulton, 1920). Of the total  $\text{CO}_2$  approximately  $\frac{19}{100}$  is in the form of bicarbonate (Van Slyke and Cullen, 1917). When the  $\text{CO}_2$  tension is other than 40 mm. the normal  $[\text{BHCO}_3]$  figures also change. For when the  $\text{CO}_2$  tension is increased the  $[\text{H}^+]$  is also increased, and the ratio  $(\text{BA}):[\text{HA}]$  for hemoglobin and the other buffers is lowered, since it changes in accordance with the equation  $\frac{[\text{BA}]}{[\text{HA}]} = \frac{K}{[\text{H}^+]}$ . ( $[\text{BA}]$  = concentration of alkali salt of buffer acid, and  $[\text{HA}]$  = concentration of free buffer acid.) In consequence of the change base from the buffers other than bicarbonate is set free and combines with  $\text{CO}_2$  to form  $\text{BHCO}_3$ . Rise of  $\text{CO}_2$  tension therefore increases not only the  $[\text{H}_2\text{CO}_3]$  but also the  $[\text{BHCO}_3]$  and *vice versa*. Consequently above and below 40 mm.  $\text{CO}_2$  tension the normal limits of blood bicarbonate and total  $\text{CO}_2$  content rise and fall as indicated by the two absorption curves of Fig. 1. (These curves are taken from Peters and Barr (1921) who plotted them to include an area containing all the apparently reliable  $\text{CO}_2$  absorption curves of normal human blood in the literature.)

As shown by L. J. Henderson (1921) the variations in the acid-base balance of a given blood are indicated by the changes in any two of a number of interdependent variables, including the buffers other than bicarbonate, the plasma chloride, and the

[HbO<sub>2</sub>] : [Hb] ratio. In Fig. 1, for example, any point may be located by any two of four values; the total CO<sub>2</sub>, the CO<sub>2</sub> tension, the pH, and the H<sub>2</sub>CO<sub>3</sub>. Of such variables, however, the bicarbonate is of peculiar significance because, as a result of the unlimited supply of H<sub>2</sub>CO<sub>3</sub>, bicarbonate is the form taken by all bases in the blood not bound by acids other than carbonic. Consequently, as will be seen later, values of [BHCO<sub>3</sub>], taken together with those of another determining factor such as the pH, yield information concerning the available alkali which might be difficult to ascertain otherwise.

*Representation of the Combined Variations in Blood Bicarbonate and Hydrion Concentration.*—The blood conditions may be represented by a diagram of the type used by Haldane and others to show the "CO<sub>2</sub> absorption curves" of the blood, and recently further elaborated by Straub and Meier (1918) and by Haggard and Y. Henderson (1919) to show also the pH values.

If we draw a curve, expressing [BHCO<sub>3</sub>] values as ordinates, and [H<sub>2</sub>CO<sub>3</sub>] values as abscissæ, the curve will be a slanting straight line for all points corresponding to any given [BHCO<sub>3</sub>] : [H<sub>2</sub>CO<sub>3</sub>] ratio, and the slant will be more or less steep according as the [BHCO<sub>3</sub>] : [H<sub>2</sub>CO<sub>3</sub>] ratio is great or small. But a constant [BHCO<sub>3</sub>] : [H<sub>2</sub>CO<sub>3</sub>] ratio indicates a constant pH (see equation of Hasselbalch below). Consequently, we are able by a series of straight, slanting lines on a diagram arranged as described to express all possible [BHCO<sub>3</sub>] : [H<sub>2</sub>CO<sub>3</sub>] ratios and pH values. In pure NaHCO<sub>3</sub> - H<sub>2</sub>CO<sub>3</sub> solutions, the isohydrionic lines curve slightly, because the proportion of NaHCO<sub>3</sub> dissociated into Na<sup>+</sup> and HCO<sub>3</sub>' increases slightly with dilution. In blood, where the Na<sup>+</sup> concentration is constant, the lines are practically straight. Their slope may be calculated from the equation of L. J. Henderson (1909),  $[H^+] = K_1 \frac{[H_2CO_3]}{[BHCO_3]}$ , or by the same equation in the logarithmic form used by Hasselbalch (1916),  $pH = pK_1 + \log \frac{[BHCO_3]}{[H_2CO_3]}$ .  $K_1 = \frac{K}{\lambda}$ , K being the dissociation constant of carbonic acid,  $\lambda$  the degree of dissociation of BHCO<sub>3</sub> into B<sup>+</sup> and HCO<sub>3</sub>'.  $pK_1$  is the negative logarithm of K<sub>1</sub>. The value of K<sub>1</sub> for blood was estimated by Haggard and Henderson (1919), from the data available in the literature, as  $8 \times 10^{-8}$ , for which

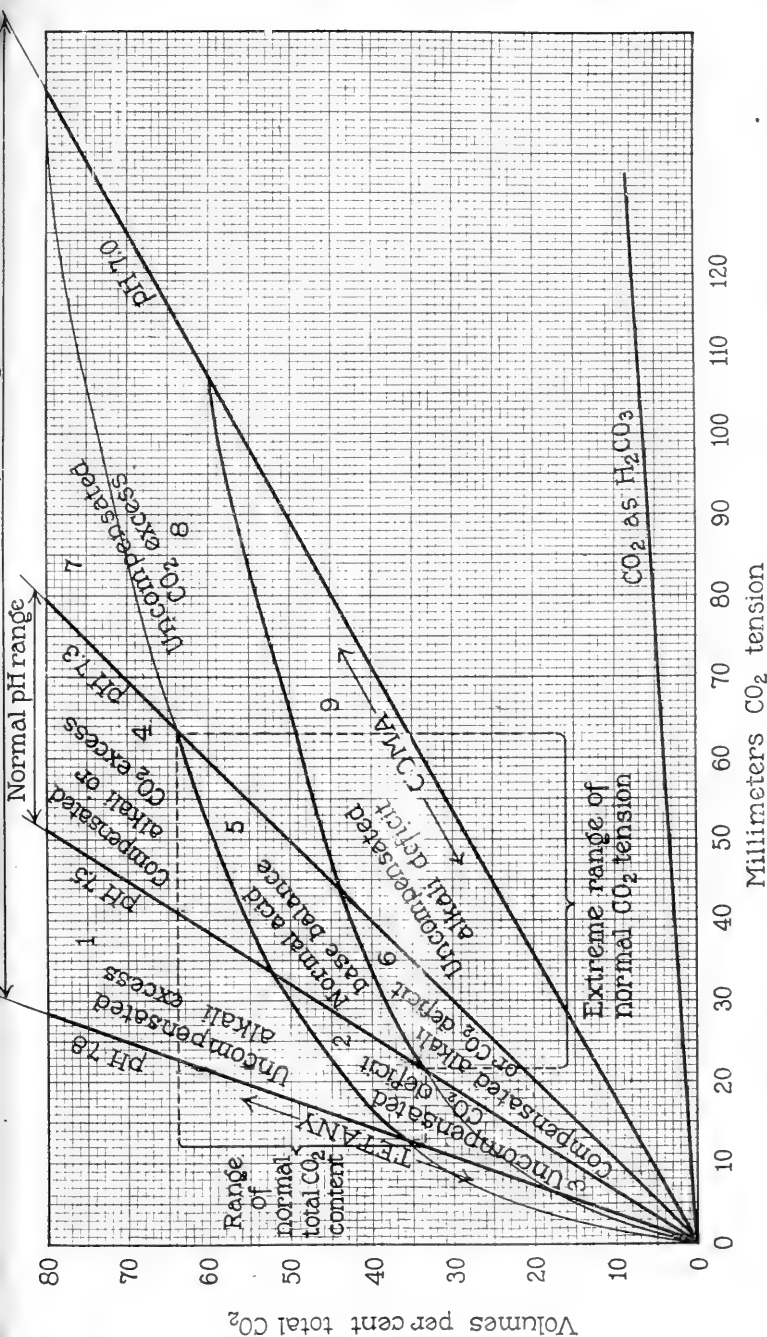


Fig. 1. Normal and abnormal variations of the  $[H_2CO_3]$ ,  $[H_2CO_3]$ ,  $CO_2$  tension, and pH in oxygenated human whole blood drawn from resting subjects at sea-level. The bicarbonate  $CO_2$  at any point is obtained by subtracting from the total  $CO_2$  the relatively small amount present as  $H_2CO_3$  indicated by the slanting line near the bottom of the figure. (See discussion of Area 5.)

For blood in which the hemoglobin is completely reduced, Christiansen, Douglas, and Ilaldane (1914) have shown that the absorption curves are higher than in oxygenated blood by about 6 volumes per cent of  $CO_2$  at ordinary  $CO_2$  tensions.

the negative logarithm, and therefore the corresponding value of  $pK_1$ , is 6.10.

The equation  $pH = 6.10 + \log \frac{[BHCO_3]}{[H_2CO_3]}$  has accordingly been used in plotting the pH lines of Fig. 1 from which Figs. 2 and 3

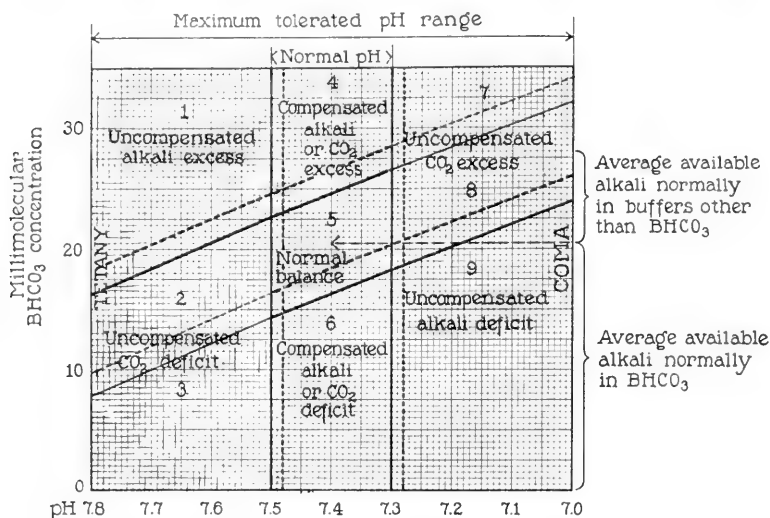


FIG. 2. Normal and abnormal variations of the  $[BHCO_3]$  and pH values in arterial and venous human whole blood. The arterial conditions are indicated by the solid curves, venous by the broken curves.  $[BHCO_3]$  and pH values are represented with rectangular coordinates, and the diagram, as compared with Fig. 1, is simplified by omitting  $[H_2CO_3]$  and  $CO_2$  tension values.  $[BHCO_3]$  values are expressed in terms of millimolecular concentration (1 millimolecular  $[BHCO_3] = 2.24$  volumes per cent of bicarbonate  $CO_2$ ).

are derived. That the value 6.10 will be subject to correction in the second decimal place as the result of further work appears probable, but it is sufficiently accurate to serve our present purposes.

For Fig. 1, since it was desired to use the customary form of  $CO_2$  absorption curves, with total  $CO_2$  values,  $[BHCO_3 + H_2CO_3]$ ,

as ordinates, and  $\text{CO}_2$  tensions as abscissæ, the form of the equation used was  $\text{pH} = 6.10 + \log \frac{(\text{total } \text{CO}_2) - (0.0672p)}{0.0672p}$ ,  $p$  being the  $\text{CO}_2$  tension in mm. of mercury, 0.0672 the factor by which the tension is converted into terms of volumes per cent of  $\text{CO}_2$  physically dissolved (as  $\text{H}_2\text{CO}_3$ ) in the blood (Bohr, 1905).

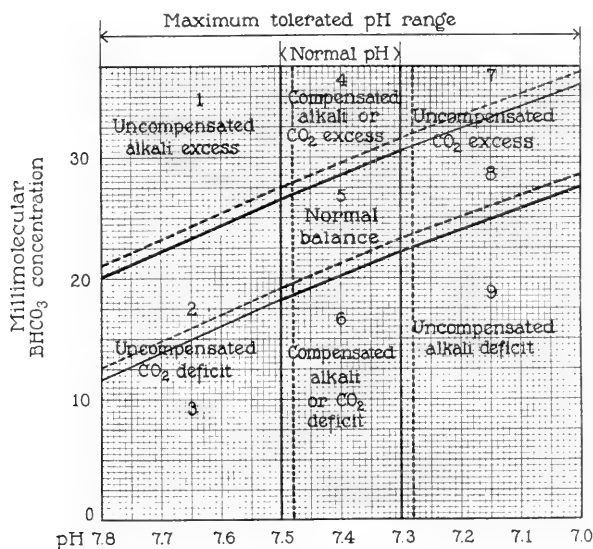


FIG. 3. Normal and abnormal variations of the  $[\text{BHCO}_3]$  and pH in serum or oxalate plasma. Arterial conditions are indicated by the solid curves, venous by the broken curves. The curves are 4 millimols higher than those of Fig. 2, since the  $\text{BHCO}_3$  concentration in the plasma at any given pH is higher than that of the whole blood by approximately 10 volumes per cent of bicarbonate  $\text{CO}_2$ , or 4 millimols of  $\text{BHCO}_3$  per liter. The venous bicarbonate curves are only half as far from the arterial as in the case of whole blood, since the data of Joffe and Poulton (1920), and of Smith, Means, and Woodwell (1921) indicate that the  $[\text{BHCO}_3]$  difference between venous and arterial blood is less in the plasma than in the cells.

*The Combined Variations of Blood Bicarbonate and pH, and the Conditions Associated with Them.*

As stated above, the possible conditions may be classified as those in which the blood bicarbonate is high, low, or normal, and combined with each of these may be a high, low, or normal pH, so that thus classified there are nine possible different conditions of the acid-base balance. These conditions, represented by the nine areas shown in Fig. 1, are the following:

*Area 1.—Uncompensated Alkali Excess.*—In this condition the  $[\text{BHCO}_3]$  is increased above the normal without a parallel increase in  $[\text{H}_2\text{CO}_3]$ . The result is an increase in the  $[\text{BHCO}_3]:[\text{H}_2\text{CO}_3]$  ratio and therefore in pH. That is, the blood reaction becomes overalkaline.

The condition appears to occur after overdosing with sodium bicarbonate (Howland and Marriott, 1918; Harrop, 1919; Davies, Haldane, and Kennaway, 1920). It has also resulted from loss of gastric HCl caused by obstructing the pylorus and regularly washing out the stomach for some days (MacCallum, Lintz, Vermilye, Leggett, and Boas, 1920).

It is accompanied by an increase in alveolar  $\text{CO}_2$  tension, due to a slowing of respiration in the apparent attempt of the organism to hold back sufficient  $\text{CO}_2$  to restore to normal the overalkaline reaction. (If this compensation is accomplished the condition shifts to that represented by Area 4.) There is a moderate diuresis, and bicarbonate is excreted in the urine at a rate that may be several grams per hour (Davies, Haldane, and Kennaway). Ammonia almost completely disappears from the urine and the titratable acid may become a negative quantity. The cessation of acid excretion and its replacement by alkali excretion tend to reduce the  $[\text{BHCO}_3]$  to normal, and bring the system back, probably through Area 4, to the normal condition represented by Area 5.

The most marked and characteristic clinical effect of uncompensated alkali excess is the development of symptoms of tetany when the alkalization proceeds sufficiently far. From this fact, however, one is not at present justified in assuming that all tetany is either caused by, or accompanied by, alkalosis. McCallum and his coworkers did not find high plasma bicarbonate

in tetany caused by parathyroidectomy, although McCann (1918) did find it.

*Areas 2 and 3.—Uncompensated CO<sub>2</sub> Deficit.*—In this condition the  $[\text{H}_2\text{CO}_3]$  is decreased without a parallel fall in  $[\text{BHCO}_3]$ . The result is, therefore, as in the condition represented by Area 1, an increase in the  $[\text{BHCO}_3]:[\text{H}_2\text{CO}_3]$  ratio and the pH, but it is due to loss of  $[\text{H}_2\text{CO}_3]$  instead of increase in  $[\text{BHCO}_3]$ .

Area 2 represents the first result of lowering in blood  $[\text{H}_2\text{CO}_3]$  by a respiratory stimulus other than either the blood hydron or  $\text{H}_2\text{CO}_3$  concentration. The consequence is an overalkaline reaction.

A compensating retention of acid metabolites, indicated by a decreased excretion of ammonia and titratable acid in the urine sets in, and there is also, as when the pH is raised by increased  $[\text{BHCO}_3]$ , an excretion of bicarbonate (Davies, Haldane, and Kennaway). As a result of these compensatory processes the bicarbonate of the blood may be lowered in some hours from Area 2 to Area 3 (partial compensation), and eventually to Area 6, where the pH is again down to its normal value (entire compensation). This last condition is attained when one becomes acclimated to a high altitude (Hasselbalch and Lindhard, 1915).

Uncompensated CO<sub>2</sub> deficit has been caused in man by hyperpnea either voluntary (Collip and Backus, 1920; Davies, Haldane, and Kennaway, 1920; Grant and Goldman, 1920; Milroy, 1914), or induced by breathing air with a diminished oxygen content, such as is encountered at high altitudes (Haggard and Henderson, 1920; Haldane, Kellas, and Kennaway, 1919). Bazett and Haldane (1921) have observed it as the result of hyperpnea caused by emersion in warm water. Apparently unusual demands on the lungs for either oxygenation or cooling may arouse respiratory stimuli which to some extent rob the hydron stimulus of its usual control.

The effects of uncompensated CO<sub>2</sub> deficit on the urine are similar qualitatively to those of uncompensated alkali excess; there is a decrease in ammonia excretion, an increase in urinary pH, and an excretion of bicarbonate (Davies, Haldane, and Kennaway). The rate of bicarbonate excretion observed, however, was much less (only a fraction of a gram per hour) when the blood pH was raised by overbreathing than when it was raised by administration of bicarbonate.

The ultimate clinical symptoms are again those of tetany

and have been identified as such by Grant and Goldman (1920). The characteristic signs after voluntary deep breathing for an hour or less included the carpopedal spasm, Chvostek's sign, Trousseau's sign, Erb's sign, and in one instance even a tetanic convulsion. The physiological effects of abnormally high blood pH appear to be similar, whether the increase is caused by an increase in the numerator or a fall in the denominator of the  $[\text{BHCO}_3]: [\text{H}_2\text{CO}_3]$  ratio.

Area 4.—Compensated  $\left\{ \begin{array}{l} \text{Alkali Excess or} \\ \text{CO}_2 \text{ Excess.} \end{array} \right.$ —Here the pH is normal,

the  $[\text{BHCO}_3]$  is high but is balanced by a proportionally high  $[\text{H}_2\text{CO}_3]$ . The state of the acid-base balance of the blood is the same, whether the original disturbance is alkali retention (Area 1) or  $\text{CO}_2$  retention (Areas 7 and 8). Hence the condition may be described as either compensated alkali excess or compensated  $\text{CO}_2$  excess, according to whether the primary disturbance is due to alkali or  $\text{CO}_2$  retention. The condition indicated by Area 4 has been observed to arise from both causes.

*Alkali excess* has been observed after therapeutic overadministration of sodium bicarbonate. If, as is usually the case following moderate oral administration, the absorption is not rapid,  $\text{CO}_2$  may be retained sufficiently to balance the increased  $[\text{BHCO}_3]$ , and the condition changed from that indicated by Area 5 merely to that of Area 4. If absorption is too rapid for simultaneous compensation by  $\text{CO}_2$  retention, the condition changes to that indicated by Area 1, presumably to return later through Area 4 to normal Area 5.

*Compensated  $\text{CO}_2$  excess* appears to be the state observed by Scott (1920) in emphysema. The retarded gas exchange presumably leads to a state of chronically increased  $\text{CO}_2$  tension in the blood, and the body raises the blood  $[\text{BHCO}_3]$  high enough to balance the  $[\text{H}_2\text{CO}_3]$  and maintain a normal reaction.

It appears that this condition, primarily due to  $\text{CO}_2$  retention, may be differentiated from that in which alkali retention is the primary cause, by the fact that the former is associated with cyanosis (as in emphysema), either permanent or caused by slight exertion. Diffusion of oxygen is so much slower than that of  $\text{CO}_2$  (Krogh, 1919) that any hindrance retarding the alveolar gas exchange sufficiently to affect  $\text{CO}_2$  excretion would presumably be accompanied by still more hindrance to oxygenation of the



blood. This presumption is further supported by the work of Krogh and Krogh (1910) who found in rabbits that while  $\text{CO}_2$  tension in arterial blood and alveolar air are equal, oxygen tension is lower in the blood than in the alveolar air, even when respiration is unhindered.

*Area 5.—Normal Acid-Base Balance.*—The normal area represents the balance that is practically always found in the resting individual in health and at ordinary altitudes. (At higher altitudes the normal dissociation curve falls parallel with the barometric pressure (Y. Henderson, 1920)). Area 5 covers approximately the conditions represented in detail by the nomogram of L. J. Henderson (1921).

The minimum and the maximum normal arterial  $\text{CO}_2$  tensions and bicarbonate concentrations indicated by the lower left and upper right corners of Area 5 are about twice as far from the means as are the normal extremes for these values heretofore estimated from alveolar air and arterial blood analyses. The wide range indicated by the diagram may be due to the fact that technical errors have widened in all directions the ranges indicated by the boundaries of Area 5. More accurate data will perhaps show this area to be smaller, and the extremes therefore less far apart. For the pH limits of the plasma Parsons is inclined to place the normal range at more nearly between pH 7.30 and 7.40 than the doubly wide range of 7.30 to 7.50 which we have allowed.

Another reason in part perhaps responsible for the fact that the extreme  $[\text{BHCO}_3]$  and  $\text{CO}_2$  tension values of Area 5 exceed the normally observed extremes is that there would be only one chance out of many for maximum pH and minimum  $[\text{BHCO}_3]$ , or the reverse, to occur in the same individual; *e. g.*, if levels of each so far from the mean are taken as to include only 1 individual out of 20, presumably only 1 out of 400 would show both extremes at once. Consequently it would not be surprising if the extreme normal limits of  $\text{CO}_2$  tension and  $[\text{BHCO}_3]$  have hitherto escaped observation, or have been observed so rarely as not to be regarded normal.

With more accurate technique and a larger number of observations it appears probable that the limits of normal arterial  $\text{CO}_2$  tension and bicarbonate concentration indicated by a graphic estimation like the above will coincide with those observed.

Area 6.—Compensated  $\left\{ \begin{array}{l} \text{Alkali Deficit or} \\ \text{CO}_2 \text{ Deficit.} \end{array} \right.$  —Area 6 represents a

condition in which the available blood alkali is lowered, but in which a normal pH is maintained because the fall in  $[\text{BHCO}_3]$  is balanced by a proportional fall in  $[\text{H}_2\text{CO}_3]$ . The primary cause of the condition may be a fall in either  $[\text{H}_2\text{CO}_3]$  or  $[\text{BHCO}_3]$ , decrease in the other factor being in each case a secondary balancing or compensatory process, with the apparent physiological object of maintaining a normal pH. Although the final state of the acid-base balance in the blood is in each case the same, *viz.* proportionally lowered  $[\text{BHCO}_3]$  and  $[\text{H}_2\text{CO}_3]$  with normal pH, the state may nevertheless with some advantage be differentiated by two terms, as either compensated alkali deficit or compensated  $\text{CO}_2$  deficit, according to whether the primary cause of the abnormality is deficit in the available alkali, caused by retention or overrapid formation of non-volatile acids, or whether it is deficit in  $[\text{H}_2\text{CO}_3]$  caused by some respiratory stimulus added to or intensifying the usual  $[\text{H}^+]$  stimulus. In alkali deficit the result of a failure in the secondary compensating processes would be acidification, in  $\text{CO}_2$  deficit it would be alkalinization. Presumably different means, depending on the nature of the primary cause, may be required in each case to restore and maintain normality.<sup>1</sup>

*Compensated alkali deficit* is the condition occurring as the result of accelerated production of non-volatile acids (diabetes) or their retarded elimination (presumably the case in nephritis). The bicarbonate reserve of the entire body is diminished, that of the blood falling parallel with that of the other body fluids. In experimental intoxication of rabbits with  $\text{HCl}$ , Goto (1918) has found that the potassium phosphate of the tissues and the  $\text{CaCO}_3$  of the bones are also reduced.

In the evident attempt to maintain a normal  $[\text{H}_2\text{CO}_3]$ :  $[\text{BHCO}_3]$  ratio and pH in the blood, ventilation becomes deeper, and the  $[\text{H}_2\text{CO}_3]$  is reduced in proportion to the  $[\text{BHCO}_3]$ . Presumably there is during the acid invasion a slight increase in hydron concentration, causing the blood condition to shift toward the border which separates Areas 6 and 9. The respiratory center is, however, at once stimulated, and  $\text{CO}_2$  is driven off so

<sup>1</sup> Whether the lowered blood alkali of traumatic shock is primarily due to non-volatile acid retention (Cannon, 1918) or to  $\text{CO}_2$  loss (Y. Henderson and Haggard, 1918) is still uncertain.

that until compensation breaks down pH is kept within normal limits and the condition remains in Area 6. At the same time accelerated formation of ammonia and excretion of buffer acids, such as acid phosphate, tend to raise the available alkali back to normal.

Compensated alkali deficit is the condition commonly observed as the result of retention of non-volatile acids in metabolic diseases, such as diabetes and nephritis (discussed by Van Slyke and Cullen, 1917) and marasmus of infants (Schloss and Harrington, 1919; Howland and Marriott, 1916). Until recently it has been the only form of naturally occurring acidosis recognized clinically, except the uncompensated acidosis (Area 9) of the premortal state. Since the pH is normal, the alkali neutralized by the invading acids is solely that of the bicarbonate (see page 169), and the fall in blood bicarbonate is an exact measure of the non-volatile acid that enters the blood.

For the reason that this condition at the time represented all clinically observed acidoses, except the uncompensated premortal acidosis, in which also, however, the bicarbonate is reduced, Van Slyke and Cullen in 1917 defined acidosis as a condition in which the blood bicarbonate is lowered. The definition is adequate for the forms of acidosis caused by retention of non-volatile acids, but it does not cover the conditions since observed which are represented by Areas 7 and 8, and which are caused by CO<sub>2</sub> retention; and it fails to exclude the conditions represented by Areas 2 and 3, in which the bicarbonate is reduced as a result not of acid retention but of CO<sub>2</sub> loss. With apparent adequacy, however, one may define acidosis as a condition caused by acid retention sufficient to lower either the bicarbonate or the pH of the blood below the normal limits.

*Compensated CO<sub>2</sub> Deficit.*—As already stated above, a fall of blood alkali with maintenance of normal pH may also occur when the primary cause is not acid retention, but excessive respiratory loss of CO<sub>2</sub>. In this case the fall in blood bicarbonate is a compensatory process which tends to prevent the blood reaction from becoming abnormally alkaline. One respiratory stimulant which has been demonstrated to have such an effect is oxygen want.

Y. Henderson (1920) has shown from data obtained by Fitzgerald, and by Douglas, Haldane, Henderson, and Schneider

(1912) on their Pike's Peak expedition that the  $\text{CO}_2$  of the alveolar air is lowered at high altitudes, and varies in direct proportion to the barometric pressure. This is also shown by the data of Hasselbalch and Lindhard (1915). Since the rate of  $\text{CO}_2$  production is not lowered (Hasselbalch and Lindhard), it is evident that the minute volume of air breathed is increased, an effect which is also noted when air with reduced oxygen percentage is breathed at sea-level pressure.

The process by which the state of compensated  $\text{CO}_2$  deficit is reached has been outlined in the discussion of the condition represented by Areas 2 and 3.

The final effect, lowered bicarbonate with normal pH, is the same as in compensated retention of non-volatile acids. The primary cause, however, is not acid retention, but loss of an acid (carbonic) which is compensated by a reduction of the blood alkali.

*Areas 7 and 8.—Uncompensated  $\text{CO}_2$  Excess.*—In this condition respiratory excretion of  $\text{CO}_2$  is retarded, either by physical hindrance or by deadening of the respiratory center, so that the  $[\text{H}_2\text{CO}_3]$  of the blood is raised. In consequence the  $[\text{BHCO}_3]:[\text{H}_2\text{CO}_3]$  ratio and the pH are lowered. The actual blood reaction becomes less alkaline than normal.

This condition has been caused experimentally by breathing air which contains 3 to 5 per cent of  $\text{CO}_2$  (Hasselbalch and Lunds-gaard, 1912; Davies, Haldane, and Kennaway, 1920). It appears to be caused also when the respiratory center is deadened by morphine narcosis (Michaelis and Davidoff, 1912; Henderson and Haggard, 1918).

Means, Bock, and Woodwell (1921) report an observation of the condition in a cyanotic pneumonia patient.

The physiological effects are seen in an accelerated excretion of ammonia and titratable acid by the urine, the same as when the acid-base balance is shifted towards the acid side by retention of non-volatile acids. Davies, Haldane, and Kennaway (1920) observed a doubling of the rate of ammonia and titratable acid excretion after breathing air containing up to 5 per cent of  $\text{CO}_2$ . There is also, as in non-volatile acid retention, an increase in the minute volume of air expired in the apparent attempt to get rid of the excess of  $\text{CO}_2$ , unless the respiratory center is deadened, as by morphine.

The first effect of  $\text{CO}_2$  retention on the blood is to increase the  $[\text{H}_2\text{CO}_3]$  and  $[\text{H}^+]$  of the blood, without changing the buffer alkali content (condition represented by Area 8). Thus Davies, Haldane, and Kennaway found after breathing for an hour air containing  $\text{CO}_2$  in amounts gradually increasing up to 5 per cent, there was no change in the  $\text{CO}_2$  capacity of the blood (unchanged total buffer alkali). Henderson and Haggard (1918) found in dogs a rise of 2 or more volumes per cent in  $\text{CO}_2$  capacity of the blood within a half hour after injecting morphine, or breathing air containing 5 or more per cent of  $\text{CO}_2$ .

The increase in the blood alkali occurring in Area 7 is secondary to the  $[\text{H}_2\text{CO}_3]$  increase and is compensatory in its nature; it tends to raise the  $[\text{BHCO}_3]:[\text{H}_2\text{CO}_3]$  ratio back to normal by increasing the  $[\text{BHCO}_3]$  to balance the increased  $[\text{H}_2\text{CO}_3]$ . In consequence the blood condition from Area 8 shifts to Area 7 (partial compensation). Normal Area 5 is presumably regained either by passing directly back to Area 5 from Area 8 (in case the excess  $\text{CO}_2$  is blown off before secondary rise of alkali to Area 7 occurs), or by passing through Areas 7 and 4 to 5 (in case compensation is accomplished partly by the secondary rise in alkali).

The compensatory increment of blood alkali in Area 7 probably comes from two sources: (1) The increased excretion of ammonia and titratable acid through the kidneys tends to raise the bicarbonate content of the entire body, and the blood plasma bicarbonate would normally rise with that of the other fluids. (2)  $\text{HCl}$  may perhaps leave the blood plasma and enter the tissue cells, as it has been shown to leave the plasma and enter the blood cells when the pH rises (for discussion and literature on this electrolyte shift see Van Slyke, 1921). The rate of blood alkali rise observed by Henderson and Haggard appears too rapid to be probably accounted for by acid excretion alone, and these authors attribute the increase to alkali drawn from the tissues. The effect would be the same if acid passes from the blood into the tissues, a process which from analogy with the shift between plasma and blood cells seems more probable. The relative parts that these two factors, acid excretion and shift of acid to the tissues (or of alkali in the reverse direction), play in the compensatory rise of blood bicarbonate during  $\text{CO}_2$  retention is uncertain. That accelerated acid excretion occurs has been shown. That acid shift from blood to tissues also occurs seems probable.

*Area 9.—Uncompensated Alkali Deficit.*—In this condition the  $[\text{BHCO}_3]$  of the blood is lowered without a proportional fall in  $[\text{H}_2\text{CO}_3]$ . In consequence there is a fall in the  $[\text{BHCO}_3]:[\text{H}_2\text{CO}_3]$  ratio and the pH.

This is the condition defined by Hasselbalch and Gammeltoft (1915) as "uncompensated acidosis." It has been most frequently observed in cases of nephritic and diabetic acidosis in the premortal period. Means, Bock, and Woodwell (1921) describe both the symptoms and blood changes in such a nephritic case. The blood bicarbonate is extremely low. Respiration, which up to the terminal stage has kept the  $[\text{H}_2\text{CO}_3]$  sufficiently low to maintain a normal  $[\text{H}_2\text{CO}_3]:[\text{BHCO}_3]$  ratio, now fails to do so, and the blood condition shifts from that represented by Area 6 over into that represented by Area 9.

In deep ether anesthesia, according to the results of Van Slyke, Austin, and Cullen (1919-20), the blood state is represented by Area 9, and both alkali deficit and carbonic acid retention occur. This combination appears also to occur in some cardiac cases (Peters and Barr, 1921).

*Relation of Changes in the Acid-Base Balance of the Blood to  
Changes in the Other Body Fluids.*

As has already been shown, the intercellular fluids other than blood plasma have, so far as studied, been found under normal conditions to approximate the blood plasma in bicarbonate and hydrion concentrations. There is evidence that in changes from the normal the other body fluids follow more or less promptly the blood plasma. Van Slyke and Cullen (1917) found that when acid was injected into the circulation the fall in blood bicarbonate was only about one-sixth as great as it would have been had the acid all remained in the blood; the other five-sixths of the acid must have gone into the other body fluids and the tissues, or drawn alkali from them. Palmer and Van Slyke (1917) found similarly that when bicarbonate was administered the rise in blood bicarbonate was approximately that calculated on the assumption that the alkali was not retained in the blood, but was distributed evenly through all the body fluids. Collip and Backus (1920) found that the bicarbonate of the spinal fluid follows that of the venous blood plasma. When the latter was lowered by

continued etherization, or by shock (handling of intestines) the bicarbonate of the spinal fluid also fell and to about the same level, although more slowly. When the bicarbonate of the blood was raised by bicarbonate injection, the spinal fluid bicarbonate rose in the course of a few hours to approximately the same level.

*The Physiologically Available Alkali of the Bicarbonate and of the Other Blood Buffers.*

In order that a buffer shall neutralize an acid (HCl for example) *without change in pH* it is necessary that the buffer acid HA, set free by the reaction  $BA + HCl = BCl + HA$ , shall be completely removed, and in addition as much of the HA formerly present as may be necessary to keep the ratio  $[BA]:[HA]$  at the original value. Of the blood's important buffers, plasma protein, cell phosphates, hemoglobin, and bicarbonate, only the bicarbonate has an acid which can be quickly removed. Under nearly all circumstances, physiological and pathological, in which the respiratory apparatus is not specifically affected, it appears that the  $[H_2CO_3]$  is so regulated that a normal pH is maintained. This is accomplished even by the ill diabetic or nephritic so efficiently that, until the  $[BHCO_3]$  has been reduced by invading acids to one-fourth, and perhaps even one-eighth of its normal value, the  $[H_2CO_3]$  is reduced in the same proportion, and a normal pH is maintained.

So long as the pH is kept constant in the above manner, all the changes in buffer alkali are those of the bicarbonate. For at a constant pH the ratio  $[BA]:[HA]$  remains constant for each buffer, in accordance with the general equation for salts of weak acids; viz.,  $\frac{[HA]}{[BA]} = K_1 [H^+]$ . Consequently, however much depletion the  $[BHCO_3]$  may suffer, the alkali salts of the other buffers are unaffected.

However, when a fall in pH occurs, the alkali of the other buffers is drawn upon and, as mentioned before, conditions have been recently observed in which a fall in pH does occur. From the data available it appears that for a short time at least the pH may fall as low as 7.0, although not much lower without fatal results.

The maximum available alkali of the blood is therefore almost the entire alkali of the bicarbonate plus that portion of the other buffer alkalies which is yielded when the pH changes from normal to the minimum compatible with life. The amount of available buffer alkali may be estimated by increasing the  $\text{CO}_2$  tension of the blood until the pH is reduced from 7.4 to 7.0. All the alkali given up by the other buffers is bound by  $\text{H}_2\text{CO}_3$  and thereby turned into bicarbonate under these conditions, so that the increase in bicarbonate above that at normal  $\text{CO}_2$  tension and pH 7.4 represents the available alkali of the other buffers. By extrapolation of the average normal  $\text{CO}_2$  absorption curve of human blood in Fig. 2 we find that this increase covers the range from 20.5 to 28.0 millimolecular  $[\text{BHCO}_3]$ . The available alkali from buffers other than bicarbonate is therefore 7.5 millimolecular in concentration, equivalent to  $2.24 \times 7.5 = 17$  volumes per cent of  $\text{CO}_2$ , or approximately one-third the normal blood bicarbonate alkali.

The average total alkali of normal human blood available for neutralizing invading acids may therefore be summarized as:

0.0205 M bicarbonate alkali, (equivalent to 46 volumes per cent of bicarbonate  $\text{CO}_2$ ). Of this three-fourths, and perhaps seven-eighths, may be used for neutralization of acid without change in pH and most of the remainder becomes available if the pH falls to 7.0.

0.0075 M alkali, (equivalent to 17 volumes per cent of bicarbonate  $\text{CO}_2$ ), from other buffers, available only when the pH falls to 7.0.

Total 0.280 M available alkali, (equivalent to 63 volumes per cent of bicarbonate  $\text{CO}_2$ ).

Of the 0.0075 M alkali available from buffers other than bicarbonate the greater part is normally bound to hemoglobin (Van Slyke, 1921)<sup>2,3</sup>

<sup>2</sup> p. 160.

<sup>3</sup> If the amounts of alkali bound to oxyhemoglobin at pH 7.4 and 7.0 are calculated by the equation,  $\frac{[\text{BHCO}_3]}{[\text{HHCO}_3]} = \text{pH} - \text{pK}$ , placing  $[\text{BHCO}_3 + \text{HHCO}_3]$  equal to 0.045 M and pK, for hemoglobin equal to 7.2 (Van Slyke, 1921), we calculate in fact that the oxyhemoglobin in normal blood would yield 0.010 M alkali in changing from pH 7.4 to 7.0. This is  $\frac{1}{3}$  more than all the buffers together yield according to the curves of Fig. 2. The quantitative discrepancy will presumably disappear when the constants for hemoglobin are more accurately determined, and perhaps also when the normal  $\text{CO}_2$  absorption curves in the pH range 7.3 to 7.0 are more accurately worked out.



In a broad sense, therefore, the alkali reserve of the blood includes not only the bicarbonate, but in addition about one-third as much alkali from the other blood buffers. In a still broader sense one might add the alkali of the bicarbonate and other buffers in the tissues and body fluids outside the circulation, since it also becomes available when the blood is flooded with acid (Van Slyke and Cullen, 1917).<sup>4</sup> In the sense, however, that it contains the only alkali that can neutralize acids without fall in blood pH, the bicarbonate forms a reserve in a class by itself.

*Means for Determining State of the Acid-Base Balance.*—It is not the purpose of the present paper to discuss in detail the technique for studying the acid-base balance of the blood and the body, but the principles appear derivable from the preceding discussion.

In order to determine which one of the possible variations exists in the blood *in vivo* it is necessary to ascertain two of the involved variables, such as the pH,  $[\text{BHCO}_3]$ , and  $[\text{H}_2\text{CO}_3]$ . With any two of them a point can be located in its proper area on a diagram such as Fig. 1, 2, or 3, but with any one of them alone it cannot be done.<sup>5</sup>

Under most conditions, pathological as well as normal, the pH is kept normal. When this is the case determination of either the  $\text{CO}_2$  tension or the bicarbonate in either plasma or whole blood suffices to indicate the condition. The plasma bicarbonate determination by the gasometric method of Van Slyke and Cullen (1917) or the titration method of Van Slyke, Stillman, and Cullen (1919) is adequate for this case, and therefore suffices for the study of metabolic conditions (*e.g.* those usually met in

<sup>4</sup> p. 338.

<sup>5</sup>When the blood is only partially saturated with oxygen, accurate location of its point on a diagram such as Fig. 1, based on figures from complete oxygenated blood, will involve a correction for the oxygen unsaturation. (The effect of oxygenation and reduction of hemoglobin in the blood bicarbonate was discovered by Christiansen, Douglas, and Haldane (1914) and has been discussed theoretically by L. J. Henderson (1920) and by the writer (1921).) For a diagram such as Fig. 1, with  $\text{CO}_2$  contents as ordinates and  $\text{CO}_2$  tensions as abscissæ, Peters and Barr (1921) have estimated the approximate correction as -0.34 volume per cent of  $\text{CO}_2$  for each volume per cent of oxygen unsaturation.

diabetes, nephritis, and metabolic disturbances of infants) in which the source of acid-base disturbance is retention of non-volatile acids, while the respiratory control of the blood reaction is unaffected. It is not adequate to cover conditions in which the respiratory control of the blood reaction is so disturbed that the pH becomes abnormal, as happens in anesthesia.

Joffe and Poulton (1920) and Peters and Barr (1921) have suggested, as the preferable single blood determination, the CO<sub>2</sub> content of the whole blood determined after equilibration with air containing CO<sub>2</sub> at 40 mm. tension. This estimation is sufficient to indicate whether the available alkali is normal or abnormal, but to indicate the entire state of the acid-base balance it is inadequate. Thus from reference to Fig. 1 it is evident that a CO<sub>2</sub> capacity of 30 volumes per cent determined at 40 mm. CO<sub>2</sub> tension on whole blood indicates a bicarbonate reserve about 12 volumes per cent (in CO<sub>2</sub> terms) below the minimum normal. Whether the condition existing in the body is that indicated by Area 3, 6, or 9, is, however, left uncertain.

In regard to the use of such diagrams as have been employed in this paper a conclusion indicated by results of Hasselbaleh, of Parsons, and of Peters and Barr may be reiterated; *viz.*, that in order to draw the most accurate deductions concerning the variations in the acid-base balance of a given individual it may be necessary to know the conditions that are normal for his particular blood. For each individual Area 5 of our diagram presumably shrinks to a fraction of the area, indicated on the charts in this paper, required to include the normal variations of the species.

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## PREPARATION AND ANALYSIS OF ANIMAL NUCLEIC ACID.

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(Received for publication, July 7, 1921.)

In recent years a number of publications have appeared dealing with the problem of animal nucleic acids. The greatest part of the work was done by Steudel and by Feulgen, and more recently there appeared a publication by Thannhauser. The work from the Berlin laboratory was done during the years of the war and became accessible to us in original form only recently.

Some of the work of Feulgen<sup>1</sup> and the recent publication of Thannhauser deal with the problem of the mode of linking of the nucleotides in the tetranucleotide. Our tentative view on this part of the problem is expressed in another article, and we shall not refer to it here.

Other articles deal with the problem of the occurrence of animal nucleic acids of two new types. The acids of one type contain only three instead of the four nucleotides, thus the new type of acids from this view-point are not tetra- but trinucleotides. It was claimed to have been isolated from the pancreas gland. On the other hand Feulgen claimed to have isolated a more complex nucleic acid containing besides the hexose nucleotides also a pentose nucleotide; namely, guanylic acid. We shall refer to these acids as mixed nucleic acids. The evidence adduced by Feulgen and later by Hammarsten<sup>2</sup> in favor of the existence of mixed nucleic acids did not seem to us convincing.

<sup>1</sup> Feulgen, R., *Z. physiol. Chem.*, 1913, lxxxviii, 370; 1917, c, 241; 1917-18, ci, 288. Feulgen, R., and Landmann, G., *Z. physiol. Chem.*, 1918, cii, 262. Feulgen, R., *Z. Physiol. Chem.*, 1919-20, cviii, 147.

<sup>2</sup> Hammarsten, E., *Z. physiol. Chem.*, 1920, cix, 141.

Also the evidence in favor of the existence of a trinucleotide in the pancreas seemed to us to require further corroboration.

Recently we were forced to resume the preparation of animal nucleic acid required for our work since the technical production of the substance in Europe has been discontinued. This necessity gave us the occasion to repeat some of our older work, with the result that we have improved somewhat our older method of preparation of the nucleic acids and the method of analysis of the purine bases in nucleic acids. In the main the method of preparation is the same as previously used by us; the new details are given in the experimental part.

For the analysis of the purine bases it was found convenient to employ alcoholysis instead of hydrolysis.

If nucleic acid is suspended in methyl alcohol and hydrochloric acid gas is passed through the solution the nucleic acid is rapidly dissolved and soon the purine bases begin to separate in the form of their hydrochloric acid salts. The separation is practically completed in 2 hours. The heat developed by the absorption of the gas is sufficient to bring about the desired cleavage.

The bases are colored very little and are readily isolated for analysis. The acids were obtained from three organs: spleen, pancreas, and liver. These three organs were selected for the reason that they are all known to contain guanylic acid together with the animal nucleic acid.

From the spleen nucleic acid, the present writer obtained a nucleic acid<sup>3,4</sup> which contained the bases in the following proportions: adenin 8.17 per cent, guanin 9.15 per cent, cytidin 7.0 per cent, and thymin 8.0 per cent. The theory for a tetra-nucleotide requires, respectively, 9.56, 10.72, 7.86, and 8.93 per cent. In a recent paper, Steudel<sup>5</sup> writes that the presence of a true nucleic acid in the spleen is still in need of proof. His method of proving it is not more rigorous than the one employed by the previous writer.

The substances now prepared had the following composition:

<sup>3</sup> Levene, P. A., *Z. physiol. Chem.*, 1905, xlv, 370.

<sup>4</sup> Levene, P. A., and Mandel, J. A., *Biochem. Z.*, 1908, x, 215.

<sup>5</sup> Steudel, H., *Z. physiol. Chem.*, 1921, cxiv, 255.



	C	H	N	P
Spleen nucleic acid.....	36.15	4.07	15.52	9.71
Pancreas " ".....	36.20	4.76	15.26	9.58
" " ".....	35.07	5.43	14.95	9.59
Liver " ".....	36.75	4.65	11.65	10.60
" " ".....	37.08	4.96	11.51	10.31
Theory for a hexosetetranucleotide....	36.30	4.19	14.79	8.73

None of the samples showed the presence of pentose and all, even the samples from the liver, contained the two purine bases. The yield of the bases was smaller, however, from the pancreas and liver nucleic acids. They were as follows:

From:	Adenin picrate.	Crude guanin.
	<i>gm.</i>	<i>gm</i>
Spleen nucleic acid.....	16.0	10.0
Pancreas " ".....	9.0	7.5
Liver " ".....	8.0	7.6

It is obvious that the elementary composition of amorphous substances of the nature of nucleic acids, which are never free from impurities, is of comparatively little service for the purpose of forming theories of molecular structure. However, great deviations from the theory, such as are observed on liver nucleic acid, require an explanation. Also, the fact that the pancreas nucleic acid, which in its elementary composition does not differ much from the spleen nucleic acid and yet furnishes on hydrolysis less purine bases than the other, needs further explanation. Work on these problems is now in progress.

#### EXPERIMENTAL.

*Preparation of Nucleic Acids.*—This is uniform for all three of the tissues, and the details of the procedure are as follows:

2,500 gm. of minced fresh tissue (previously freed from fat) are taken up in 3,000 cc. of water. 300 gm. of sodium chloride are added and all is kept boiling (with a steam coil) for 4 hours. Then 80 gm. of sodium acetate and 60 cc. of a 33 per cent solution of sodium hydroxide are added, and the mixture is allowed to

stand over night. The mixture is then neutralized with acetic acid and treated with picric acid as long as a precipitate forms. To the filtrate of this mixture hydrochloric acid is added until it turns slightly opalescent and the nucleic acid is precipitated with a 10 per cent solution of copper chloride. The copper salt of the nucleic acid is filtered and converted into the free acid by treatment with a 5 per cent solution of hydrochloric acid. The treatment is repeated once. The resulting free nucleic acid is redissolved in a 5 per cent solution of sodium hydroxide, the solution is made acid with acetic acid and precipitated with 95 per cent alcohol, containing 4 per cent of hydrochloric acid. The precipitate is then washed with 95 per cent alcohol until the washing no longer shows the presence of chlorine ions. The nucleic acid is then washed with absolute alcohol and ether, and dried. The substance so obtained does not show the presence of even traces of biuret-giving substances, and gives a negative test with orcin.

#### *Alcoholysis.*

10 gm. of nucleic acid are suspended in 200 cc. of dry methyl alcohol and hydrochloric acid gas is passed in a lively stream. After 2 hours the reaction is interrupted and the flask is allowed to stand over night. Guanin and adenin are separated in the usual way.

Guanin was purified by conversion into the sulfate, which again was converted into the free base. Adenin picrate for purification was twice recrystallized out of 10 per cent acetic acid and then dissolved in water by the addition of the required quantity of ammonia and precipitated by means of acetic acid.

#### *Analysis of Nucleic Acids.*

##### *Spleen Nucleic Acid.*

0.9880 gm. of the substance gave on combustion 0.1310 gm. of  $\text{CO}_2$  and 0.0360 gm. of  $\text{H}_2\text{O}$ .

0.0884 gm. of the substance employed for Kjeldahl nitrogen estimation required for neutralization 9.8 cc. of 0.1 N acid.

0.1768 gm. of the substance gave 0.0616 gm. of  $\text{Mg}_2\text{P}_2\text{O}_7$ .

	Calculated for $C_{13}H_{19}N_{15}P_4O_{22}$ . per cent	Found. per cent
C.....	36.30	36.15
H.....	4.19	4.07
N.....	14.79	15.52
P.....	8.83	9.71

*Bases.*—10.0 gm. of this material yield 1.6 gm. of adenin picrate and 1.0 gm. of guanin. The guanin was analyzed as the free base.

0.2000 gm. of the substance employed for Kjeldahl nitrogen estimation required 66.30 cc. of 0.1 N acid for neutralization.

	Calculated for $C_8H_5N_5O$ . per cent	Found per cent
N.....	46.53	46.41

The adenin picrate analyzed as follows:

0.0946 gm. of the dry substance employed for Kjeldahl nitrogen estimation (reduced with zinc metal) required for neutralization 21 cc. of 0.1 N acid.

	Calculated for $C_8H_5N_5.C_6H_2(NO_2)_3OH$ . per cent	Found. per cent
N.....	30.71	31.06

### *Pancreas Nucleic Acid.*

A. 0.1056 gm. of the dry substance on combustion gave 0.1358 gm. of  $CO_2$  and 0.0572 gm. of  $H_2O$ .

B. 0.1080 gm. of the dry substance of a second sample gave 0.1434 gm. of  $CO_2$  and 0.0460 gm. of  $H_2O$ .

A. 0.1818 gm. of the substance employed for Kjeldahl nitrogen estimation required for neutralization 19.40 cc. of 0.1 N acid.

B. 0.1780 gm. of another sample required for neutralization 19.40 cc. of 0.1 N acid.

A. 0.2726 gm. of the substance gave 0.0938 gm. of  $Mg_2P_2O_7$ .

B. 0.2670 gm. of another sample gave 0.0918 gm. of  $Mg_2P_2O_7$ .

	Calculated for $C_{43}H_{59}N_{15}P_4O_{32}$ . per cent	Found. per cent	
		A	B
C.....	36.30	35.07	36.02
H.....	4.19	5.43	4.76
N.....	14.79	14.95	15.26
P.....	8.73	9.59	9.58

*Bases.*—The substance was alcoholized in 10.0 gm. lots. The average yield was 0.900 gm. of adenin picrate and 0.750 gm. of crude guanin. Guanin was analyzed as the free base and gave the following values.

0.1066 gm. of the substance gave 0.1566 gm. of  $\text{CO}_2$  and 0.0310 gm. of  $\text{H}_2\text{O}$ .

0.0996 gm. of the substance gave 39.4 gm. nitrogen gas at  $T = 26^\circ$  and  $P = 756$  mm.

	Calculated for $\text{C}_5\text{H}_5\text{N}_3\text{O}$ . per cent	Found. per cent
C.....	39.73	39.05
H.....	3.30	3.26
N.....	46.60	46.62

Adenin picrate analyzed as follows:

0.0935 gm. of substance employed for nitrogen estimation (modified Kjeldahl) required for neutralization 20.25 cc. of 0.1 N acid.

	Calculated for $\text{C}_5\text{H}_5\text{N}_3 \cdot \text{C}_6\text{H}_2(\text{NO}_2)_3\text{OH}$ . per cent	Found. per cent
N.....	30.71	30.32

#### *Liver Nucleic Acid.*

A. 0.9720 gm. of the dry substance gave 0.1330 gm. of  $\text{CO}_2$  and 0.0434 gm. of  $\text{H}_2\text{O}$ .

B. 0.1090 gm. of another sample gave 0.1390 gm. of  $\text{CO}_2$  and 0.0566 gm. of  $\text{H}_2\text{O}$ .

A. 0.0900 gm. of the dry substance employed for Kjeldahl nitrogen estimation required for neutralization 7.40 cc. of 0.1 N acid.

B. 0.1816 gm. of another sample required for neutralization 13.60 cc. of 0.1 N acid.

A. 0.1800 gm. of the dry substance gave 0.0666 gm. of  $\text{Mg}_2\text{P}_2\text{O}_7$ .

B. 0.2724 gm. of another sample gave 0.1108 gm. of  $\text{Mg}_2\text{P}_2\text{O}_7$ .

	Calculated for $\text{C}_{43}\text{H}_{59}\text{N}_{15}\text{P}_4\text{O}_{32}$ . per cent	Found. per cent	
		A	B
C.....	36.30	37.08	34.79
H.....	4.19	4.96	5.81
N.....	14.79	11.51	10.48
P.....	8.73	10.31	11.33

*Bases.*—One lot of 50.0 gm. was treated in methyl alcohol and hydrochloric acid gas as described above. This yield was 3.8 gm. of crude guanin and 4.0 gm. of crude adenin picrate.

Guanin was identified as the free base and analyzed as follows:

0.0997 gm. of the base gave on combustion 0.1566 gm. of  $\text{CO}_2$  and 0.0338 gm. of  $\text{H}_2\text{O}$ .

	Calculated for $\text{C}_5\text{H}_5\text{N}_3\text{O}$ . <i>per cent</i>	Found. <i>per cent</i>
C.....	39.73	39.55
H.....	3.30	3.47

Adenin was identified as picrate and analyzed as follows:

0.0940 gm. of the substance employed for nitrogen estimation (modified Kjeldahl) required for neutralization 20.50 cc. of 0.1 N acid.

	Calculated for $\text{C}_5\text{H}_5\text{N}_3 \cdot \text{C}_6\text{H}_2(\text{NO}_2)_3\text{OK}$ . <i>per cent</i>	Found. <i>per cent</i>
N.....	30.71	30.53



## THE LIVER LECITHIN.

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(Received for publication, July 7, 1921.)

The older work on the unsaturated lipoids of the liver has been reviewed in the paper of Levene and Ingvaldsen. In the same paper a new analysis of the liver lecithin was reported. The new facts reported in that paper related principally to the nature of the fatty acids. In the course of the work reported by Levene and Ingvaldsen<sup>1</sup> two fatty acids were found, one saturated, and the other unsaturated which analyzed for a polyunsaturated arachidic acid. Since an acid of that structure has never before been described in connection with lecithin, further corroboration of the finding seemed desirable. Furthermore, in the course of the present year it was shown by Levene and Rolf<sup>2</sup> that the egg lecithin and that of the brain contained two saturated fatty acids. This result was obtained by means of fractional distillation of the esters of the combined saturated acids. In light of this observation it became necessary to reinvestigate all lecithins in regard to the number of fatty acid radicles, saturated and unsaturated, present in their molecule. In regard to the liver lecithin it was now found that it contained two saturated and two unsaturated acids.<sup>3</sup>

The saturated acids are palmitic and stearic. They were isolated and identified by the same procedure as described by Levene and Rolf.

The unsaturated acids are: one, unsaturated stearic, and the other, unsaturated arachidic. On reduction one is converted into stearic and the other into arachidic. The exact degree of

<sup>1</sup> Levene, P. A., and Ingvaldsen, T., *J. Biol. Chem.*, 1920, xliii, 359.

<sup>2</sup> Levene, P. A., and Rolf, Ida P., *J. Biol. Chem.*, 1921, xlvi, 193, 353.

<sup>3</sup> Evidence has recently been obtained in this laboratory that egg lecithin also contains the two unsaturated acids.

unsaturation of either one of the two acids is as yet not known. There are however, indications that one (arachidic) may be tetra-unsaturated. On the addition of bromine a substance was obtained which analyzed for an octobromide of arachidic acid. However, it will require a larger quantity of material to establish the degree of unsaturation of each of the two acids with certainty. The presence of several acids in the liver lecithin again emphasizes the question of the existence of more than one lecithin.

It was attempted to answer this question by the molecular weight estimation of the hydrolecithin. The hydrolecithin from the liver lecithin has been prepared essentially according to Paal's procedure.

The molecular weight of the substance was found 810 and 700 (in two estimations). The theory of a monophosphatide requires 809, that of a diphosphatide 1600. Consequently, liver lecithin consists of a mixture of monolecithins.

In the course of the present work the process of preparation of pure free lecithin from its cadmium chloride salt has been improved so that analytically pure substance is prepared in good yield; namely, about 50 gm. of free lecithin from 100 gm. of the cadmium chloride salt.

The procedure in the main is as follows: The salt of lecithin is dissolved in chloroform and this solution is transferred into a solution of dry ammonia gas in dry methyl alcohol. The resulting lecithin is purified from the slight quantity of impurities by the acetic acid process developed by Levene and Ingvaldsen. The details of the procedure are given in the experimental part.

#### EXPERIMENTAL.

##### *I. Preparation of Pure Lecithin.*

Various attempts to produce pure liver lecithin without converting it into the cadmium chloride salt met with little success. The following method proved to be the easiest and most efficient.

The liver, in 100 pound lots, is minced, dried, and extracted, first with acetone, second with ether, and last with alcohol. These extracts are treated separately as follows:

*Acetone Extract.*—This is allowed to stand at 0°C. over night. A precipitate of fat is deposited, which is removed by filtration.



The filtrate is concentrated (if necessary) and the lecithin precipitated by adding a saturated solution of cadmium chloride in alcohol until no further precipitate is formed.

The residue from the above filtration is suspended in alcohol and warmed until the fat is melted. The mixture is then cooled over night. The fat precipitated on standing is again filtered from the alcoholic solution and again treated with alcohol as before. This extraction is repeated until the mother liquor no longer gives with cadmium chloride a precipitate of lecithin cadmium salt. The latter is recognized by the fact that on dissolving in a small amount of moist ether it is again precipitated by the addition of an excess of acetone. From three to seven extractions may be required. The alcoholic mother liquors are then precipitated with cadmium chloride.

*Ether Extract.*—This is concentrated to a small volume and allowed to stand at 0°C., when a precipitate consisting of fat and cerebrosides is formed. The precipitate is extracted with ether. The ethereal extract is added to the original filtrate and cooled once more to permit the separation of the cerebrosides which the solution may still contain. After filtering, the combined mother liquors are concentrated and treated with alcohol to separate the lecithin from cephalin. The alcoholic liquors are then treated with cadmium chloride.

*Alcoholic Extract.*—This is likewise concentrated and cooled to remove cerebrosides, the mother liquor being decanted if possible, otherwise filtered, or centrifuged if necessary.

The cerebrosides are again extracted with warm ether. The extract is cooled and centrifuged. The alcoholic and ethereal liquors are then treated with cadmium chloride.

#### *Treatment of Cadmium Chloride Salts.*

It is necessary to allow the cadmium chloride precipitate of lecithin to stand at least half an hour until it is sufficiently coagulated to permit filtration. The filtered material, which is not quite dry, is transferred to a large beaker or precipitating jar and stirred up with a large volume of cold acetone. If the acetone liquor turns dark from dissolved material the suspension is allowed to settle, the liquor decanted off, and more cold acetone is added. Finally the material is filtered by suction.

This material is purified in two steps: the one, is the "ether crystallization," the other is the "toluene-ether" process.<sup>1</sup> It is a matter of judgment as to which shall be used first and the number of times which each should be repeated. The aim is to obtain a white granular material which filters quickly.

*The ether crystallization* consists in dissolving the cadmium chloride salt in warm ether, water being added, a few drops at a time, until the suspended material goes into solution. An excess of water hinders the solution of the larger particles. The solution is allowed to stand over night, or longer, at 0°C. The substance should separate in a granular form, easily filterable by suction. If it forms a pasty solution not easily filtered, time and material will be wasted in attempting a filtration. Another precipitation with acetone should remove impurities which interfere with the process.

This purification removes not only the fats and oils but also takes out most of the cephalin present. Since the cadmium chloride salt is itself slightly soluble in cold ether, some of the material may be lost in purification. Hence the following precautions are necessary. 1. Excess of ether is to be avoided. With very impure material it is more advisable to repeat the purification several times with small quantities of solvent than to use a large excess at one time. The amount of ether filtered off should not be more than twice the volume of the residue. 2. The filtered material should *not* be washed with ether, but should be filtered as quickly as possible until the solvent runs very slowly. 3. In case the material fails to filter properly, it should be transferred to a beaker, warmed slightly until dissolved, precipitated with acetone, purified by the toluene-ether method, and subsequently passed through the ether crystallization process. 4. The filtration should be carried out in the cold.

*The toluene-ether* process consists in dissolving the cadmium chloride salt in a minimum volume of toluene (adding a slight amount of water if necessary). If the toluene fails to dissolve all the material the residue should be centrifuged off. The solution is then treated with 4 volumes of ether containing 1 per cent water. The solution is cooled to 0°C. over night and filtered.

The latter method gives larger yields but removes less of the cephalin and other impurities. It probably removes impurities not taken out by the former method, hence the cadmium chloride compound should be purified by both methods.

Experience shows that in the case of liver lecithin the toluene-ether method should precede the ether crystallization method of purification of the cadmium chloride salt in order to obviate difficulty in filtering from the ether.

One purification by each method should be sufficient to give almost white dry material with an amino content of less than 3 per cent of the total nitrogen present. Such a product may be converted into free lecithin.

*Conversion of the Cadmium Chloride Compound into Free Lecithin.*

The cadmium chloride salt is dissolved in chloroform and is converted into free lecithin by means of a solution of ammonia in methyl alcohol. 100 gm. of the cadmium chloride salt are dissolved in 300 cc. of warm chloroform and poured into 400 cc. of methyl alcohol containing 20 gm. of ammonia gas. This is added slowly with rapid stirring. The product of reaction is allowed to stand a short time before filtering. The precipitate may be filtered off through a folded filter paper. The chloroform methyl alcohol solution of lecithin is then concentrated under diminished pressure. Near the end of the concentration the material foams considerably for a short time and then the foaming subsides. The vacuum concentration should be carried out at a low temperature. If during the operation a precipitate of fat settles out this should be filtered off. The remaining lecithin is practically free from solvent. It is dissolved in a minimum (5 to 10 cc.) of glacial acetic acid. This is poured into 800 cc. of boiling hot acetone, stirred, and allowed to cool to room temperature. A very small dark precipitate (1 to 2 gm.) settles out. The supernatant liquid is decanted or filtered. The precipitate is slightly soluble in ether and insoluble in acetone but somewhat soluble in ethyl alcohol and more soluble in methyl alcohol.

No. 126.

0.0154 gm. of substance gave on combustion 0.0954 gm. of  $H_2O$ , 0.2315 gm. of  $CO_2$ , and 0.0114 gm. of ash.

0.1910 gm. of substance used for Kjeldahl nitrogen determination required 3.90 cc. of 0.1 N acid corresponding to 0.00546 gm. of N.

0.2865 gm. of substance gave 0.0390 gm. of  $\text{Mg}_2\text{P}_2\text{O}_7$ .

$\text{C}_{44}\text{H}_{87}\text{O}_9\text{NP}$ . Calculated. C 65.59, H 10.89, N 1.74, P 3.86.

Found. C 61.16, H 10.34, N 2.92, P 3.88.

It contains 10 per cent amino nitrogen.

No. 124.

1.5 gm. of substance were hydrolyzed with HCl, neutralized, concentrated, and made up to 15 cc.

5 cc. of this solution required for Kjeldahl nitrogen determination 5.60 cc. of 0.1 N HCl.

2 cc. of this solution for Van Slyke determination gave 0.57 cc. of  $\text{N}_2$  at  $T = 27^\circ$ ,  $P = 762.2$  mm.

$$\frac{\text{Amino N}}{\text{Total N}} = \frac{10}{100}$$

The liquors are then cooled in a freezing mixture to  $-5^\circ\text{C}$ . Frequently at this phase a second small precipitate settles out. A sample of this material analyzed as follows:

No. 122.

0.1024 gm. of substance gave on combustion 0.1098 gm. of  $\text{H}_2\text{O}$ , 0.2216 gm. of  $\text{CO}_2$ , and 0.0118 gm. of ash.

0.1832 gm. of substance for Kjeldahl nitrogen determination required 3.10 cc. of 0.1 N acid corresponding to 0.00434 gm. of N.

0.2748 gm. of substance gave 0.0464 gm. of  $\text{Mg}_2\text{P}_2\text{O}_7$ .

$\text{C}_{44}\text{H}_{87}\text{O}_9\text{NP}$ . Calculated. C 65.59, H 10.89, N 1.74, P 3.86.

Found. C 59.50, H 12.10, N 2.37, P 4.75.

The mother liquors are concentrated under diminished pressure until all the ether and most of the acetic acid are removed. Water is added a little at a time and the material is shaken or stirred until a thick emulsion of a light brown color is formed. This is poured into 800 to 1,000 cc. of acetone, chilled down to  $-5^\circ\text{C}$ . It is carefully stirred and allowed to stand at 0 to  $-5^\circ\text{C}$ . over night, when it is transferred to a crystallizing dish and washed free from excess water by stirring with cold dry acetone. The acetone is decanted off and the lecithin dried in a vacuum desiccator.

From 40 to 45 gm. of pure material may be obtained from 100 gm. of cadmium chloride salt. (Theoretical yield, 81 to 82 gm.) Several samples have been analyzed. They differed little one from another in their elementary composition. The analysis of one of them is as follows:

No. 119.

0.0996 gm. of substance gave on combustion 0.0994 gm. of  $H_2O$ , 0.2768 gm. of  $CO_2$ , and 0.0090 gm. of ash.

0.1798 gm. of substance required 2.40 cc. of 0.1 N acid, corresponding to 0.00336 gm. of N.

0.2697 gm. of substance gave 0.0390 gm. of  $Mg_2P_2O_7$ .

$C_{44}H_{87}O_9NP$ .\* Calculated. C 65.59, H 10.89, N 1.74, P 3.86.

Found. C 64.83, H 11.16, N 1.87, P 4.03.

\* This formula represents material consisting of equal parts of two lecithins, each one containing two of the four fatty acids.

## II. The Fatty Acids of Lecithin.

For the preparation of fatty acids from lecithin, the material was hydrolyzed 8 to 15 hours with 10 parts of 10 per cent HCl. The fatty acids on cooling appeared as a semisolid cake. They were dissolved in methyl alcohol and precipitated in the presence of ammonium hydroxide with a nearly equal weight of lead acetate dissolved in a minimum quantity of water. After freezing, the mother liquors were filtered off. The lead salts which contained both the saturated and unsaturated fatty acids were extracted repeatedly with boiling ether until further extraction produced only slight precipitate with hydrochloric acid.

The ether solution then contained the lead salts of the unsaturated acids while the ether-insoluble material consisted of the lead salts of the saturated acids. Both fractions were decomposed with HCl, dissolved in ether, washed with water, dried, and the solvent evaporated off.

A lot of 528 gm. of the lecithin cadmium chloride free from amino nitrogen was hydrolyzed with 10 per cent solution of hydrochloric acid. The yield of fatty acids was 223 gm.

### *Unsaturated Fatty Acids.*

These were obtained by extracting the lead salt first by means of acetone and then by means of ether. Each extract was worked over separately. The acetone extract was concentrated and the residue thus obtained extracted with ether. From both of these fractions the acids were liberated and reconverted into the lead salts. These were again purified and again converted into the free acids. A sample of the acids gave the following iodine and hydrogen values.

0.2907 gm. of substance absorbed 0.435 gm. of iodine by the Wijs method.  
 0.5141 gm. of substance reduced by Paal's method absorbed 67 cc. of  $H_2$  in 3 hours at  $17^\circ C.$ , 759 mm. pressure, or 1.103 gm. of  $H_2$  per 100 gm. of substance.

$C_{18}H_{34}O_2$ .	Calculated.	Iodine value	91,	Hydrogen number	0.721.
$C_{18}H_{32}O_2$ .	Calculated.	" "	182,	" "	1.447.
	Found.	" "	154,	" "	1.103.

It was later found that this material consisted of two fatty acids, hence it is possible that one was a singly unsaturated, and that the other contained two or more double bonds.

The free fatty acids were finally reduced by Paal's method. The samples of reduced acids obtained from each fraction analyzed as follows:

No. 84 (material obtained from the acetone extract of the lead salts).  
 0.1020 gm. of substance gave on combustion 0.1198 gm. of  $H_2O$  and 0.2830 gm. of  $CO_2$ .

No. 85 (material obtained from the ether extract of the lead salts).

0.1012 gm. of substance gave on combustion 0.1190 gm. of  $H_2O$  and 0.2828 gm. of  $CO_2$ .

No. 84. C 75.96, H 13.19.

" 85. C 76.21, H 13.15.

Since the two fractions proved practically of identical elementary composition they were combined and converted into the methyl esters. These were freed from adhering sulfuric acid by washing with water and finally by recrystallization from methyl alcohol. They were then fractionated by distilling at a pressure of 1 to 2 mm.

The following fractions were obtained.

A.....	182-185°C.
B.....	175-185°C.
C.....	182-195°C.
D.....	185-203°C.

Fractions A and D were redistilled and the following fractions were obtained.

From A	A <sub>1</sub> .....	158-165°C.
	A <sub>2</sub> .....	170-182°C.
From D	D <sub>1</sub> .....	182-192°C.
	D <sub>2</sub> .....	187-197°C.

For identification, the esters were saponified with an alcoholic solution of sodium hydroxide. The acids were liberated and converted into the lead salts. The acids were again liberated from the lead salts and analyzed. Fraction A<sub>1</sub> corresponded apparently to pure stearic acid.

Analysis 101.

0.1024 gm. of substance gave on combustion 0.1186 gm. of H<sub>2</sub>O and 0.2860 gm. of CO<sub>2</sub>.

0.8950 gm. of substance in a molecular weight determination required 6.50 cc. of 0.5 N NaOH.

C<sub>18</sub>H<sub>36</sub>O<sub>2</sub>. Calculated. C 75.93, H 12.76.

Found. C 76.16, H 12.96.

Molecular weight was 275, that of stearic acid is 284.

The substance melted at 70.5–71°C., stearic acid melts at 70–71°C.

When this was mixed with some very pure stearic acid melting at 74°C., the mixture melted at 74°C.

Fraction D<sub>2</sub> apparently corresponded to pure arachidic acid.

Analysis 100.

0.1000 gm. of substance gave on combustion 0.1166 gm. of H<sub>2</sub>O and 0.2822 gm. of CO<sub>2</sub>.

0.9760 gm. of substance neutralized 6.75 cc. of 0.5 N NaOH.

C<sub>20</sub>H<sub>40</sub>O<sub>2</sub>. Calculated. C 76.95, H 12.91.

Found. C 76.97, H 13.24.

Molecular weight was 314, that of arachidic acid is 313.

The substance melted at 75.5–76°C., arachidic acid melts at 75–77°C.

When this was mixed with some pure arachidic acid melting at 75°C., the mixture melted at 75°C.

*Saturated Fatty Acids.*

The lead salts which were insoluble in acetone and ether were converted into free acids. These were twice esterified with methyl alcohol. The mixture of methyl esters thus obtained was distilled at a pressure of 1 to 2 mm. into the following fractions:

a.....	160–163°C.
b.....	159–167°C.
c.....	158–172°C.
d.....	170–180°C.

and residue.

Fractions a and d were redistilled as follows.

From a	a <sub>1</sub> .....	156-162°C.
	a <sub>2</sub> .....	Residue.
From d	d <sub>1</sub> .....	180-183°C.
	d <sub>2</sub> .....	182-188°C.

Fraction a<sub>1</sub> apparently corresponded to pure palmitic acid.

Analysis 92.

0.1009 gm. of substance gave on combustion 0.1220 gm. of H<sub>2</sub>O and 0.2802 gm. of CO<sub>2</sub>.

0.8168 gm. of substance neutralized 6.14 gm. of 0.5 N NaOH.

C<sub>16</sub>H<sub>32</sub>O<sub>2</sub>. Calculated. C 74.92, H 12.58.

Found. C 75.09, H 12.98.

Molecular weight was found to be 266, palmitic acid had a molecular weight of 256.

The melting point was 62°C., palmitic acid melts at 63-64°C.

When this was mixed with some pure palmitic acid melting at 64°C., the mixture melted at 63°C.

Fraction d<sub>2</sub> apparently corresponded to pure stearic acid.

Analysis 94.

0.1009 gm. of substance gave on combustion 0.1220 gm. of H<sub>2</sub>O and 0.2802 gm. of CO<sub>2</sub>.

0.6686 gm. of substance neutralized 4.82 cc. of 0.5 N NaOH.

C<sub>18</sub>H<sub>36</sub>O<sub>2</sub>. Calculated. C 75.93, H 12.76.

Found. C 75.72, H 13.53.

Molecular weight was 278, stearic acid had a molecular weight of 284.

The substance melted at 71°C., stearic acid melts at 70-71°C.

When this was mixed with a sample of very pure stearic acid melting at 74°C. the mixture melted at 74°C.

### III. Bromine Addition Products of the Unsaturated Acids.

An attempt was made to separate and to characterize the individual unsaturated acids by preparing the bromine addition products. 40 gm. of pure lecithin which had been prepared from the cadmium chloride salt as described above, were used.

No. 119.

0.0996 gm. of substance gave on combustion 0.0994 gm. of H<sub>2</sub>O, 0.2768 gm. of CO<sub>2</sub>, and 0.0090 gm. of ash.

0.1798 gm. of substance required 2.40 cc. of 0.1 N acid, corresponding to 0.00336 gm. of N.

0.2697 gm. of substance gave 0.0390 gm. of Mg<sub>2</sub>P<sub>2</sub>O<sub>7</sub>.

C<sub>44</sub>H<sub>87</sub>O<sub>9</sub>NP. Calculated. C 65.59, H 10.89, N 1.74, P 3.86.

Found. C 64.83, H 11.16, N 1.87, P 4.03.



This was hydrolyzed with a 10 per cent solution of hydrochloric acid, the acids were dissolved in ether, washed with water, dried, and the ether evaporated off. The iodine number of the mixed acids was 91.

0.2457 gm. of substance absorbed 0.232 gm. of iodine by the Wijs method.  
Average molecular weight of 280. Calculated. Iodine value 91.  
Found. " " 91.

The acids were converted into the lead salts, the unsaturated acids extracted with ether and converted into the free acids. These were dissolved in 18–30° petroleic ether and brominated at 0°C. with 3 cc. of bromine dissolved in petroleic ether.

On freezing to  $-10^{\circ}$  a precipitate was obtained. The mother liquor was concentrated and again cooled to  $-10^{\circ}$ . The combined precipitate was recrystallized from petroleic ether and then recrystallized from ethyl ether.

This gives three fractions: A, the petroleic ether-soluble fraction; B, the fraction insoluble in petroleic ether but soluble in ethyl ether; and C, the fraction insoluble in both solvents. This last fraction contains the material having most bromine (namely, the hexabromides and octobromides, if present). The first fraction should be largely dibromides while the tetrabromides should predominate in the fraction insoluble in petroleic ether but soluble in ethyl ether.

Fraction C (the material insoluble in both solvents) was recrystallized from ethyl ether; the yield was 1 gm. In an open tube melting point determination it darkened, turning black at 200°C. It contracted at 240°C. and decomposed at 243°C. In a closed tube it contracted at 239°C. and melted without decomposition at 243°C. This analyzed as follows:

No. 129.

0.2012 gm. of substance gave 0.2936 gm. of AgBr.

This would indicate a hexabromide.

$C_{20}H_{34}O_2Br_6$ .	Calculated for hexabromarachidic acid.	61.0.
$C_{18}H_{30}O_2Br_6$ .	Calculated for hexabromstearic acid.	63.2.
Found.		62.11.

The material was recrystallized from ether. On heating in an open tube it darkened at 180–200°C., contracted at 240°C., and decomposed at 244°C.

It analyzed as follows:

Analysis 130.

0.1068 gm. of substance gave 0.1670 gm. of AgBr.

This corresponds more closely to an octobromide.

$C_{26}H_{32}O_2Br_8$ .	Calculated for octobromarachidic acid.	67.80.
	Found.	66.55.

There was not sufficient material for further treatment.

#### IV. *Hydrolecithin from Liver Lecithin.*

For the preparation of hydrolecithin 10 gm. of pure liver lecithin free from amino nitrogen were used (Analysis 119 given above). This was reduced by Paal's method. The hydrolecithin produced was recrystallized twice from acetone and once from methyl ethyl ketone.

This analyzed as follows:

No. 128.

0.1074 gm. of substance gave on combustion 0.1082 gm. of  $H_2O$ , 0.2554 gm. of  $CO_2$ , and 0.0098 gm. of ash.

0.1926 gm. of substance for Kjeldahl nitrogen determination required 2.40 cc. of 0.1 N acid, corresponding to 0.00336 gm. of N.

0.2839 gm. of substance gave 0.0400 gm. of  $Mg_2P_2O_7$ .

$C_{44}H_{91}O_9NP$ .	Calculated.	C 65.30, H 11.33, N 1.73, P 3.84.
	Found.	C 65.03, H 11.29, N 1.74, P 3.86.

A molecular weight determination was made as follows:

1.036 gm. of substance raised the boiling point of 16 gm. methyl alcohol 0.071°C.

0.964 gm. of substance raised the boiling point of 16 gm. methyl alcohol 0.077°C.

$C_{44}H_{91}O_9NP$ .	Calculated.	Molecular weight 809.
	Found.	First determination 810.
		Second " 700.

# ON THE NUMERICAL VALUES OF THE OPTICAL ROTATIONS IN THE SUGAR ACIDS.

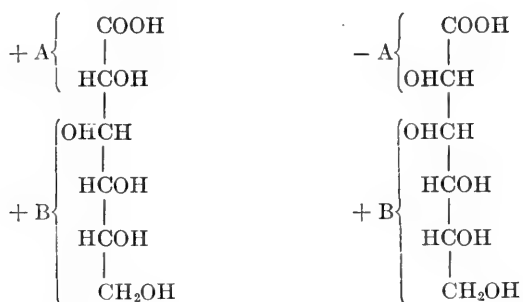
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(Received for publication, July 7, 1921.)

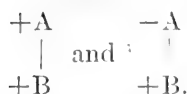
In recent years the van't Hoff theory of optical superposition has been on several occasions the subject of discussion. Some writers, Rosanoff, and independently of him Patterson and Taylor have challenged the theory, whereas in a series of very important publications Hudson<sup>1</sup> has demonstrated the validity of the theory and made it the foundation of many important contributions to the chemistry of carbohydrates.

The present writer also has made use of the theory for the purpose of establishing relationships between the configuration of the carbon atom 2 and the optical rotation of epimeric sugar acids. It was found that in a pair of epimeric sugar acids as in gluconic and mannonic acids the molecular structure may be regarded as consisting of two parts, one (A) consisting of carbon atoms 1 and 2, and the other (B) of carbon atoms 3, 4, 5, and 6 as seen from the following.



<sup>1</sup> Hudson, C. S., *J. Am. Chem. Soc.*, 1909, xxxi, 66; 1917, xxxix, 462; 1918, xl, 813.

It is evident that such pairs of epimers may be represented as



It was found that in sugar acids and in 2-hexosaminic acids the superposition theory holds to the following extent; the direction of the rotation of the carbon atom 2 is in agreement with the theory in all of the four pairs of hexonic and all of the four pairs of hexosaminic acids. The numerical value of the rotation of carbon atom 2 should according to the theory of van't Hoff be identical for all hexonic and for all hexosaminic acids. This expectation was not realized. In three of the epimers of each group the values are identical, but in the fourth it is markedly different as seen from the following table.

	$[\alpha]_D^{20}$ of carbon atom 2.	$[M]_D^{20}$	Phenylhydrazides of	$[\alpha]_D^{20}$ of carbon atom 2.	$[M]_D^{20}$
Epichitosaminic ..	+12.5	+24.37 (10 <sup>2</sup> )	Gluconic....	+14.25	+42.18 (10 <sup>2</sup> )
Chitosaminic.....	-12.5	-24.37 (10 <sup>2</sup> )	Mannonic...	-14.25	-42.18 (10 <sup>2</sup> )
Dextro-xylohexosaminic.....	+12.5	+24.37 (10 <sup>2</sup> )	Gulonic.....	+14.25	+42.18 (10 <sup>2</sup> )
Levo-xylohexosaminic.....	-12.5	-24.37 (10 <sup>2</sup> )	Idonic.....	-14.25	-42.18 (10 <sup>2</sup> )
Epichondrosaminic	+12.5	+24.37 (10 <sup>2</sup> )	Galactonic..	+ 8.25	+24.42 (10 <sup>2</sup> )
Chondrosaminic...	-12.5	-24.37 (10 <sup>2</sup> )	Talonic.....	- 8.25	-24.42 (10 <sup>2</sup> )
Dextro-ribohexosaminic.....	+19.12	+37.28 (10 <sup>2</sup> )	Allonic.....	+20.8	+61.56 (10 <sup>2</sup> )
Levo-ribohexosaminic.....	-19.12	-37.28 (10 <sup>2</sup> )	Altronic .....	-20.8	-61.56 (10 <sup>2</sup> )

Hudson was stimulated by our findings on the hexonic and hexosaminic acids and developed a more general conclusion, that the direction of the rotation of the carbon atom 2 determines the direction of the optical rotation of the acid. This is seen by mere comparison of our tables without resorting to calculation. Hudson arrived at his conclusion in a very ingenious way. He assumed that the superposition theory holds literally in every detail and he concluded that the magnitude and the direction of the rotation of any one of the four asymmetric carbon atoms

in hexonic acid can be calculated from four equations each representing the algebraic sum of the four asymmetric carbon atoms of one acid and of the value of its optical rotation in the following way.

1. *d*-Gluconic acid  $+ \alpha - \beta + \gamma + \delta = (+12.0) (286) = +34.3 (10^2)$
2. *d*-Gulonic "  $+ \alpha - \beta - \gamma + \delta = (+13.7) (286) = +39.2 (10^2)$
3. *d*-Idonic "  $- \alpha + \beta - \gamma + \delta = (-12.4) (286) = -35.5 (10^2)$
4. *d*-Galactonic "  $+ \alpha - \beta - \gamma + \delta = (+11.0) (286) = +31.5 (10^2)$

Solving these four equations, the following values are obtained:

$$\alpha = +37.3 (10^2); \beta = +3.9 (10^2); \gamma = +1.4 (10^2); \delta = -0.6 (10^2).$$

Weerman<sup>2</sup> later working on the amides of sugar acids, corroborated the conclusions of Levene<sup>3, 4, 5</sup>, and of Hudson. Hudson alone, and later with Komatsu<sup>6</sup> repeated the observations on a series of amides. They found the following values.

$$\alpha\text{-carbon} = +47.25 (10^2); \beta\text{-carbon} = -14.65 (10^2); \gamma\text{-carbon} = +0.95 (10^2); \\ \delta\text{-carbon} = -2.05 (10^2).$$

The authors then remark: "It will be noticed that the numerical values decrease as the carbon atom is further removed from the amide end. . . The alternation in the sign of the rotation of the successive carbon atoms is also noteworthy suggesting the alternation in positive and negative affinity that is often ascribed to the carbons in a chain." Inasmuch as we have found that the value of the rotation of carbon atom 2 ( $\alpha$ , according to Hudson's nomenclature) is not a constant for all acids, it was suggested that also the magnitudes of rotation of the other carbon atoms are influenced by the configuration of the adjacent carbon atoms. If that were so then the system adopted by Hudson for his calculations may be incorrect and may lead to erroneous conclusions.

We therefore calculated the magnitudes of rotation of each of the four carbon atoms in hexonic and 2-aminohexonic acids from several combinations of four equations. If the magnitudes

<sup>2</sup> Weerman, Dissertation, Amsterdam, 1916.

<sup>3</sup> Levene, P. A., and Meyer, G. M., *J. Biol. Chem.*, 1917, xxxi, 623.

<sup>4</sup> Levene, P. A., *J. Biol. Chem.*, 1916, xxvi, 367.

<sup>5</sup> Levene, P. A., and Clark, E. P., *J. Biol. Chem.*, 1921, xlvi, 19.

<sup>6</sup> Hudson, C. S., and Komatsu, S., *J. Am. Chem. Soc.*, 1919, xli, 1141.

of rotation of each carbon atom were constant the same values should be obtained from all combinations.

It will be seen from the following table that the results obtained by Hudson and his coworkers are due to special conditions and that some combinations of four equations lead to values of rotation of the  $\alpha$ -carbon atom (Hudson's nomenclature) which are lower than some one other carbon atom.

In Combinations V, VI, and VII the values for the rotation of every carbon atom remain constant. These combinations are composed of members in which the  $\alpha$ -carbon atom is constant and equal to +12.5. On the other hand, in all combinations in which at least one member had the  $\alpha$ -carbon atom of the value 19.5 the values for the other carbon atoms were variable. It seemed therefore that the values for  $\beta$ -,  $\gamma$ -, and  $\delta$ -carbon atoms may possess constant and equal values when derived from the values of Part B of each acid, and not from that of the entire acid.

Calculations again showed that the values for  $\beta$ -,  $\gamma$ -, and  $\delta$ -carbon atoms remained equal and constant when derived from values of Part B of acids having the rotation of  $\alpha$ -carbon atom = 12.5, and not otherwise.

This observation naturally leads to the conclusions that the superposition theory holds only within certain limits.

In certain substances, as in hexonic acids, the vicinity of a certain group (carboxyl) accentuates the rotation of the  $\alpha$ -carbon atom to such an extent that the direction of its rotation determines the direction of the rotation of the entire molecule. The correctness of this conclusion with certainty can be demonstrated only on a comparison of the rotation of pairs of epimers.

It is, however, interesting to note that for the series of gluconic and mannonic, galactonic and talonic, gulonic and idonic, the superposition theory holds completely.

#### Hexosaminic acid.

I		
$[\alpha]_D$	$[\alpha]_D$	$[M]_D$
$\delta + \gamma + \beta + \alpha = +12.5$	$\alpha = +11.625$	+22.65 ( $10^2$ )
$\delta - \gamma - \beta + \alpha = + 8.0$	$\beta = + 2.125$	+ 4.14 ( $10^2$ )
$\delta + \gamma - \beta - \alpha = -15.0$	$\gamma = + 0.125$	+ 2.44 ( $10^2$ )
$\delta - \gamma + \beta - \alpha = -11.0$	$\delta = - 1.375$	-26.82 ( $10^2$ )

## II

$\delta + \gamma + \beta - \alpha = -26.5$	$\alpha = + 6.75$	$+13.15 (10^2)$
$\delta - \gamma - \beta + \alpha = + 8.0$	$\beta = - 5.75$	$-11.22 (10^2)$
$\delta + \gamma - \beta - \alpha = -15.0$	$\gamma = - 4.75$	$- 9.23 (10^2)$
$\delta - \gamma + \beta - \alpha = -17.0$	$\delta = - 9.25$	$-18.04 (10^2)$

## III

$\delta + \gamma + \beta - \alpha = -26.5$	$\alpha = +16.875$	$+32.95 (10^2)$
$\delta - \gamma - \beta - \alpha = -17.0$	$\beta = - 1.375$	$- 2.68 (10^2)$
$\delta + \gamma - \beta + \alpha = +10.0$	$\gamma = - 3.375$	$- 7.38 (10^2)$
$\delta - \gamma + \beta + \alpha = +14.0$	$\delta = - 4.875$	$- 9.50 (10^2)$

## IV

$\delta + \gamma + \beta + \alpha = +12.5$	$\alpha = +19.5$	$+38.02 (10^2)$
$\delta + \gamma + \beta - \alpha = -26.5$	$\beta = + 7.0$	$+13.65 (10^2)$
$\delta - \gamma - \beta + \alpha = + 8.0$	$\gamma = - 4.75$	$- 9.23 (10^2)$
$\delta - \gamma + \beta - \alpha = -17.0$	$\delta = - 9.25$	$-18.05 (10^2)$

## V

$\delta - \gamma - \beta + \alpha = + 8.0$	$\alpha = +12.5$	$+24.38 (10^2)$
$\delta - \gamma - \beta - \alpha = -17.0$	$\beta = + 3.0$	$+ 5.85 (10^2)$
$\delta + \gamma - \beta + \alpha = +10.0$	$\gamma = + 1.0$	$+19.50 (10^2)$
$\delta - \gamma + \beta + \alpha = +14.0$	$\delta = - 0.5$	$- 0.98 (10^2)$

## VI

$\delta - \gamma - \beta + \alpha = + 8.0$	$\alpha = +12.5$	$+24.38 (10^2)$
$\delta - \gamma - \beta - \alpha = -17.0$	$\beta = + 3.0$	$+ 5.85 (10^2)$
$\delta + \gamma - \beta - \alpha = -15.0$	$\gamma = + 1.0$	$+19.50 (10^2)$
$\delta - \gamma + \beta - \alpha = -11.0$	$\delta = - 0.5$	$- 0.98 (10^2)$

## VII

$\delta + \gamma - \beta + \alpha = +10.0$	$\alpha = +12.5$	$+24.38 (10^2)$
$\delta + \gamma - \beta - \alpha = -15.0$	$\beta = + 3.0$	$+ 5.85 (10^2)$
$\delta - \gamma + \beta + \alpha = +14.0$	$\gamma = + 1.0$	$+19.50 (10^2)$
$\delta - \gamma - \beta + \alpha = + 8.0$	$\delta = - 0.5$	$- 0.98 (10^2)$

Phenylhydrazides of hexonic acids.

## VIII

	$[\alpha]_D$	$[\alpha]_D$	$[M]_D$
$\delta + \gamma + \beta + \alpha = +25.8$	$\alpha = + 9.775$	$+27.95 (10^2)$	
$\delta - \gamma - \beta + \alpha = +12.2$	$\beta = + 8.375$	$+23.93 (10^2)$	
$\delta + \gamma - \beta - \alpha = -10.5$	$\gamma = +10.625$	$+30.38 (10^2)$	
$\delta - \gamma + \beta - \alpha = -15.0$	$\delta = - 2.975$	$- 8.50 (10^2)$	

## IX

$\delta + \gamma + \beta - \alpha = -15.8$	$\alpha = +10.95$	$+31.32 (10^2)$
$\delta - \gamma - \beta + \alpha = +12.2$	$\beta = -2.65$	$-7.58 (10^2)$
$\delta + \gamma - \beta - \alpha = -10.5$	$\gamma = -0.40$	$-1.15 (10^2)$
$\delta - \gamma + \beta - \alpha = -15.0$	$\delta = -1.80$	$+5.16 (10^2)$

## X

$\delta + \gamma + \beta + \alpha = +25.8$	$\alpha = +20.8$	$+59.48 (10^2)$
$\delta + \gamma + \beta - \alpha = -15.8$	$\beta = +7.15$	$+20.44 (10^2)$
$\delta - \gamma - \beta + \alpha = +12.2$	$\gamma = -0.35$	$-1.00 (10^2)$
$\delta - \gamma + \beta - \alpha = -15.1$	$\delta = -1.80$	$-5.14 (10^2)$

## XI

Values of Part B.

$[\alpha]_D$	$[M]_D$		$[\alpha]_D$	$[M]_D$
$\delta + \gamma + \beta + \alpha = +12.5$	$+24.37 (10^2)$			
$\delta + \gamma + \beta - \alpha = -26.5$	$-51.67 (10^2)$	$\delta + \gamma + \beta = -6.75$		$-13.16 (10^2)$

## XII

$\delta - \gamma - \beta + \alpha = +8.0$	$+15.6 (10^2)$		
$\delta - \gamma - \beta - \alpha = -17.0$	$-33.15 (10^2)$	$\delta - \gamma - \beta = -4.5$	$-8.58 (10^2)$

## XIII

$\delta - \gamma + \beta + \alpha = +14.0$	$+27.3 (10^2)$		
$\delta - \gamma + \beta - \alpha = -11.0$	$-21.45 (10^2)$	$\delta - \gamma + \beta = +1.5$	$+2.93 (10^2)$

## XIV

$\delta + \gamma - \beta + \alpha = +10.0$	$+19.5 (10^2)$		
$\delta + \gamma - \beta - \alpha = -15.0$	$-29.25 (10^2)$	$\delta + \gamma - \beta = -2.5$	$-4.88 (10^2)$

Solving for XII, XIII, and XIV, the values obtained are  $\beta = +3.0$ ,  $\gamma = +1.0$ , and  $\delta = -0.5$ .

Solving for XI, XII, and XIII, the values obtained are  $\beta = +3.0$ ,  $\gamma = -4.125$ , and  $\delta = -5.625$ .



## THE PREPARATION AND STANDARDIZATION OF COLLODION MEMBRANES.

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(Received for publication, July 6, 1921.)

The use of collodion membranes in the study of diffusion phenomena was begun by Fick (1855); the closed collodion sac was devised 5 years later by Schumacher (1860). Metchnikoff, Roux, and Taurelli-Salimbeni (1896), in their researches on the vibrio of Asiatic cholera introduced these sacs into biological science. Because of their ease of preparation, availability, thinness, and ready permeability to ordinary crystalloids, they have largely replaced the older parchment paper membranes in the study of dialysis and diffusion. For a complete bibliography and literature review of this development of their use, the reader is referred to the paper of Bigelow and Gemberling (1907). Mention should be made, however, of the work of Gorsline (1903), who showed that crystalloids were not the only substances that could diffuse through a collodion membrane, for at 35°C., peptone, albumose, albumin, starch, dextrin, and certain enzymes all dialyzed through in less than 24 hours in recognizable quantities.

The first successful attempt to produce a graded series of membranes of increasing permeability was made by Bechhold (1907, 1908). By impregnating filter papers with varying percentage strengths of gelatin or glacial acetic acid collodion, he produced membranes whose permeability varied inversely with the concentration of the impregnating solution. These membranes were used as filters. With the use of pressure, often many atmospheres, substances like hemoglobin, albumin, and various inorganic colloids were filtered through. A mechanical stirring device was employed to prevent the precipitation of the colloid on the surface of the membrane; such precipitated colloid

otherwise forming a very impervious film. The higher the pressure the more prone is this superimposed film to form. In a former investigation, De Kruif and the author (1919) were unable to prevent the formation of this film in the filtration of anaphylatoxin, the membranes becoming rapidly less permeable as filtration proceeded.

Another method of increasing and regulating the permeability of collodion membranes is that of Schoep (1911). Increased permeability is secured by adding 2 to 10 per cent of glycerol to an alcohol-ether solution of Schering's celloidin; 4 per cent of castor oil is added to give the membranes elasticity. A wide range of permeability is secured by varying the amount of glycerol added. These membranes are far more permeable than those of Beechhold; very low pressures are sufficient for the filtration of substances like albumin or hemoglobin. There is little tendency to form the objectionable colloidal film on the filtering surface. De Kruif and Eggerth (1919) used them with success in filtering the greater part of the serum proteins from anaphylatoxin.

Brown (1915) developed another method of varying permeability. Collodion sacs are air-dried and then immersed in mixtures of ethyl alcohol and water; the greater the percentage of alcohol, the more they swell, and the more permeable is the resulting membrane. This method gives a valuable series, but even the most permeable members of it (those treated with 90 to 96 per cent alcohol) do not allow much diffusion of substances such as Congo red or hemoglobin; dialysis for 2 to 7 days allows only small quantities to pass through. The Brown series, excellent in its scope, needs to be extended in the direction of greater permeability.

The Schoep membranes seem to answer that need. But the Schoep membranes are made from Schering's celloidin, which cannot be purchased in the United States now. Several brands of American pyroxylin were tried in the Schoep formulas with poor success. If little glycerol was used, the membranes were very impermeable; if the amount of glycerol was increased, they were so fragile as to be useless. Considerable improvement was effected by substituting 10 per cent of acetic or lactic acid for the castor oil used by Schoep; a stronger membrane with a

considerable range of permeability was obtained. While attempting to adapt the Schoep formulas to the available pyroxylin, it was observed that merely altering the proportion of alcohol to ether in the solvent mixture sufficed to alter the permeability of the resulting membranes through a wide and easily controllable range. These membranes have the advantage of being free from castor oil, they are transparent, and stronger than those of Schoep.

A similar observation has been made by Malfitano, who states (1908) that the higher the proportion of alcohol in the solvent, the more permeable the membrane, and in 1910 he stated that Michel and Lazarus in his laboratory had demonstrated that alcohol-rich solutions give more permeable membranes than those that are ether-rich. No further details are given.

The pyroxylin used in this investigation is that manufactured by Du Pont De Nemours and Company under the name of parlodion. All of the solutions referred to in this paper contained 6 gm. of the dry collodion in 100 gm. of alcohol-ether solvent. The parts of alcohol and ether were all taken by weight. To designate the different solutions and membranes, the following nomenclature was adopted: a solution is named according to the parts by weight of alcohol it contains; one containing 50 parts of alcohol and 50 of ether is a "50 alcohol solution;" a membrane made from this solution is designated a "50 alcohol membrane."

The ethyl alcohol first used in making up the solvent was distilled over anhydrous copper sulfate; later in the work it was distilled over calcium oxide and redistilled over metallic sodium. The ether was distilled over sodium.

Alcohol-ether mixtures containing from 20 to 80 parts of alcohol by weight readily dissolve 6 per cent of parlodion; but to obtain solutions containing 10, 15, 85, and 90 parts of alcohol, it is necessary first to make a thick solution of the collodion in a portion of the solvents and later add the remainder of the alcohol or ether, as the case may be.

On adding the last part of the ether to the 10 alcohol solution of collodion, the solution has on two occasions set to a gel, which could be redissolved by adding another per cent of alcohol. On two other occasions, a white flocculent precipitate formed, which failed to redissolve. This was allowed to settle out, and the supernatant solution was used in making the membranes.

The different collodion solutions differ considerably in their viscosity, as can be seen by referring to Table I. Viscosities were determined rather roughly by noting the time in seconds required for the solutions to flow out of the same 10 cc. pipette. The 20 alcohol solution was found to be the least viscous, viscosity increasing regularly in both directions in the series.

The changes in viscosity of these solutions after a small amount of water has been added are noteworthy. The alcohol-rich

TABLE I.  
*Relative Viscosities at 20°C.*

Solution.	Anhydrous.	5 per cent of water added.	Remarks.
10 alcohol.	35	18	Transparent emulsion.
15 "	26		
20 "	20	15	Granular precipitate. Redi- solves readily.
30 "	23		
40 "	29	26	Granular precipitate. Redi- solves readily.
50 "	34		
60 "	40	41	Gelatinous precipitate. Redi- solves with difficulty.
70 "	45		
80 "	53	85*	Sets to gel in 24 hours.
85 "	64		
90 "	85	—*	Sets to gel.

\* 2 per cent of water added.

solutions are made more viscous (only 2 per cent of moisture setting the 90 alcohol solution to a firm gel) while the ether-rich solutions are made more fluid by the same agent. Several batches of 90 alcohol solutions, made up with supposedly anhydrous solvents, slowly increased in viscosity, and after several days, set to a semisolid gel; not until the last traces of water were removed from the alcohol by redistillation over sodium was a 90 alcohol solution obtained that could be kept for weeks without marked change in viscosity.

*Method of Making the Sacs.*

The collodion sacs were made by the method devised by Novy,<sup>1</sup> with a few slight modifications. A glass tube, melted down at one end to leave a small hole, is the mold. A small fragment of cigarette paper is slightly moistened and placed over the hole, where it dries quickly; a layer of collodion is painted over the paper and the end of the tube; this is allowed to dry for 20 to 30 seconds. A few cubic centimeters of collodion solution are poured into a test-tube; this is held nearly horizontal while the end of the mold tube is immersed in the collodion. The mold tube is rotated slowly, and slowly withdrawn; then it, with its covering of collodion, is thrust horizontally into a large test-tube that lies on the table before the operator, the mold tube being rotated rapidly all the time. In these experiments the membranes were always dried for 1 minute, and then immersed in water. Rotating the membrane within the large test-tube in the manner described makes the drying slower and more uniform, and cuts off air currents. It was found to be good practice never to return any unused collodion to the stock bottle.

By filling the mold tube with water and applying air pressure to the open end with the mouth, it is usually not difficult to force water between the glass and the membrane, and so easily remove the latter. The tube and the membrane should be immersed during this process. Sometimes the membranes adhere to the glass at the edge of the hole, and no amount of gentle manipulation with the fingers can free them. Increased pressure finally breaks through, but often tears the membrane. If a short rubber tube (not too thin-walled) is slipped over the end of the mold tube and filled with water, and a small cork stopper is pushed slowly down the rubber tube, the membrane can be "started" with ease and without tearing, this expedient allowing the application of a great deal of force with a minimum of displacement. By pinching on the rubber tube as the cork is withdrawn, too much negative pressure is avoided. After loosening the membrane in this way, it can be easily removed by blowing with the mouth. The ether-rich membranes (the

<sup>1</sup>A description of this method is given in the paper by Bigelow and Gemberling (1907).

20, 30, and 40 alcohol solutions), adhere very tightly to the glass and the percentage of spoiled membranes is very high unless the rubber tube and cork are used. The 20 and 30 alcohol membranes require in addition a little manipulation with the fingers at the very end to free them.

The 10 and 15 alcohol membranes adhere so tightly to the glass that they cannot be made by this method at all, nor can they be made on the inside of a test-tube. A solid glass rod was ground down so that it tapered from a diameter of 11.0 mm. at a point 8 cm. from the end to 10.6 mm. at the end itself. The end of the rod is dipped into the collodion solution to a depth of about 1 cm.; this coat is allowed to dry for 15 to 20 seconds, and then the entire membrane is made as described above. Two coats are necessary at the extreme end, otherwise this portion is so thin that it is certain to tear. After immersing in water, the membrane is removed by peeling it down from the top. For other membranes of the series, this method does not work so well as the one first described.

#### *Method of Conducting Diffusion Experiments.*

The sacs, usually prepared on the preceding day, were each attached by means of a rubber band to a glass tube about 8 cm. long; this glass tube passed through a cork which had a longitudinal groove cut in the side. The corks fitted into the necks of test-tubes of  $\frac{1}{2}$  inch diameter. Sac's made by the first method described had a diameter of 8.3 mm. in all experiments, 3 cc. of test substance were placed in each sac, which filled it to a depth of 6 cm. The volume of the dialysate was always 10 cc., this kept constant throughout the experiment. The sacs were always immersed to a depth of 6 cm. The membranes made by the second method were larger, with an average diameter of 10.8 mm. 5 cc. of test substance were used, and they were immersed to a depth of 8 cm. All of the membranes used in the diffusion of NaCl,  $\text{KH}_2\text{PO}_4$ , saccharose, and indigo carmine (Fig. 3) were made by the second method.

In the diffusion experiments performed, four or five and sometimes more sacs made from the same solution were tested each time. Thus the experiment plotted in Fig. 1 required thirty sacs, five sacs of each of six grades of permeability.

Wherever possible, quantitative determinations of the amount of test substance that had passed through the membranes were made at different time intervals and the results plotted as in Figs. 1 and 2.

*Test Substances and Method of Their Estimation in the Dialysate.*

*Two Per Cent Aqueous Sodium Chloride.*—Estimated by titration with a standard silver nitrate solution, using potassium dichromate as an indicator.

*0.2 M Potassium Dihydrogen Phosphate.*—Estimated by titration with 0.1 *N* sodium hydroxide using phenolphthalein as an indicator and titrating to a full red color.

*Saccharose, 20 Per Cent Solution.*—Estimated with the polarimeter.

*Raffinose, 8 Per Cent Solution.*—Estimated with the polarimeter.

*Indigo Carmine (Sodium Sulfindigotate), 0.2 Per Cent Aqueous Solution.*—Estimated with the colorimeter.

*Safranine, 1 Per Cent Aqueous Solution.*—Estimated with the colorimeter.

*Primary Proteose, 1 Per Cent Aqueous Solution at pH = 7.0.*—This was prepared from Difco peptone by precipitating with  $(\text{NH}_4)_2\text{SO}_4$ , and purified by reprecipitating three times with  $(\text{NH}_4)_2\text{SO}_4$  to half saturation. The proteose was estimated by adding 6 cc. of saturated  $(\text{NH}_4)_2\text{SO}_4$  to 4 cc. of the dialysate and determining the precipitate nephelometrically.

*Dialyzed Serum Protein.*—No effort was made to separate the albumin from the pseudoglobulin, but the euglobulin was removed by centrifugation. To 10 cc. of sheep serum 1.8 cc. of 0.2 *N* HCl were added; this brings the serum approximately to the optimum reaction for the flocculation of euglobulin. This was then dialyzed in a 40 alcohol membrane against distilled water. The volume was then made up to three times the original volume of the serum. In one experiment, the protein in the dialysate was precipitated with sulfosalicylic acid and determined nephelometrically; in another, the nitrogen was determined by the micro-Kjeldahl method of Folin and Wu (1919), and the protein computed.

*Oxyhemoglobin.*—Sheep cells were washed three times with isotonic salt solution. An equal volume of distilled water was added to the packed cells and cooled; then a half volume of cold ether was added and shaken. After standing in the ice box over night, the lower layer was drawn off and diluted to a volume ten times as great as that of the original packed cells. It was estimated with the colorimeter.

*Carboxyhemoglobin.*—Carbon monoxide, prepared from oxalic acid and sulfuric acid, was bubbled through a solution of oxyhemoglobin prepared as above.

*Methemoglobin.*—To the concentrated oxyhemoglobin solution was added a half volume of 10 per cent potassium ferricyanide; this was now dialyzed in a large 40 alcohol sac until the dialysate was free from ferricyanide. It was estimated colorimetrically.

*Congo Red (Grubler's).*—A 1 per cent aqueous solution was used. This has a pH of about 8.6. It was estimated colorimetrically.

In the diffusion experiments the proteose, serum protein, and hemoglobin solutions were dialyzed against a buffered phosphate solution set at pH 7.0, made by adding 29.6 cc. of 0.2 *N* NaOH to 50 cc. of 0.2 *M*  $\text{KH}_2\text{PO}_4$  and diluting to 300 cc. The other test substances were dialyzed against distilled water. An effort was made to stabilize the reaction of the Congo red solution by using a buffered dialysate, but the presence of electrolytes greatly reduces the solubility of Congo red, so this was abandoned.

Fig. 1 shows the passage of Congo red through membranes of six grades of permeability. The 85 alcohol membranes allow very rapid diffusion of this typical "non-dialyzable colloid," while the 40 alcohol membranes allow only traces to pass through in the first 2 or 3 hours. The 30 alcohol membranes, not shown on the figure, allow no diffusion in 24 hours. In 5 hours, the 85 alcohol membranes have passed twelve times as much Congo red as the 50 alcohol ones.

By referring to Table II it will be seen that the thickness of these membranes increases towards the alcohol-rich end of the series. The higher alcohol membranes cannot be made any thinner without impairing their strength; any attempt to secure uniformity of thickness in the entire series would involve thickening the ether-rich membranes to an unnecessary and undesirable extent. It is obvious that if the membranes had



all been of the same thickness, the differences between the curves shown would, if anything, be greater; certainly not less, since the alcohol-rich membranes are the thickest and at the same time the most permeable.

A 1 per cent solution of Congo red exerts an osmotic pressure which causes an increase in the volume of the dialysee. This increase in volume is greatest with the least permeable mem-

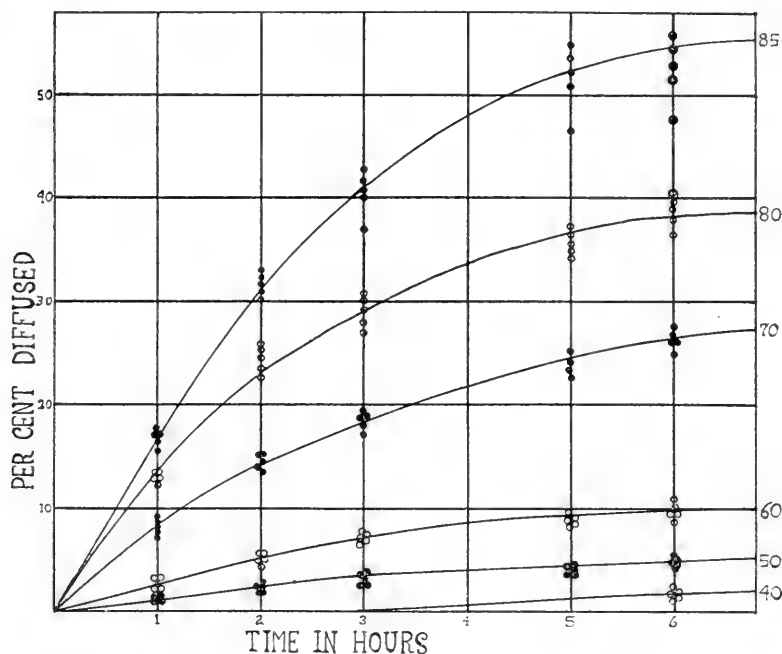


Fig. 1. Diffusion of 1 per cent aqueous Congo red at 35°C. through membranes of six grades of permeability. The numbers at the margin are the alcohol numbers of the membranes.

branes, and becomes less as permeability increases. Thus, the 40 alcohol membranes raised a column averaging 18.0 cm. in 8 hours; the 85 alcohol membranes raised a maximum column of 3.0 cm. in 3 hours, after which it began to fall. With the aid of a ruler, it was possible to place the thirty membranes used in this experiment in their correct order of permeability. Other test substances, such as hemoglobin and serum protein,

TABLE II.

Membrane.	Area = A. cm.	Empty space = r	Thickness (measured).	Thickness (calculated) = L.	Time = T. min.	Amount filtered = F. cc.	$\frac{FL}{A r T} = QL.$	$\frac{1}{L} \sqrt{\frac{FL}{A r T}}$	Wet weight. gm.	Dry weight. gm.	Ratio.
10 alcohol.	25.1	0.54	0.017	0.014	380	1.2	0.00033	0.13	0.0380	0.0220	1.73
	22.9	0.60	0.015	0.011	380	0.8	0.00017	0.11	0.0290	0.0151	1.92
	24.8	0.62	0.015	0.011	380	0.6	0.00011	0.10	0.0290	0.0148	1.95
15 "	23.7	0.71	0.011	0.010	200	1.5	0.00045	0.15	0.0250	0.0099	2.5
	23.7	0.63	0.012	0.012	200	1.1	0.00039	0.14	0.0350	0.0164	2.1
	23.7	0.62	0.011	0.011	200	1.0	0.00033	0.13	0.0330	0.0165	2.0
20 "	18.2	0.71	0.015	0.011	187	4.5	0.00210	0.21	0.0200	0.0080	2.5
	21.9	0.73	0.011	0.010	215	4.6	0.00130	0.19	0.0190	0.0070	2.7
	21.4	0.71	0.011	0.009	187	2.2	0.00075	0.17	0.0170	0.0067	2.5
30 "	23.5	0.81	0.015	0.013	54	7.1	0.00890	0.31	0.0240	0.0072	3.3
	23.0	0.78	0.016	0.013	90	7.2	0.00580	0.28	0.0230	0.0070	3.3
	19.2	0.77	0.014	0.011	102	6.7	0.00490	0.26	0.0200	0.0064	3.1
40 "	19.8	0.82	0.020	0.021	34	6.7	0.02500	0.40	0.0370	0.0095	3.9
	22.2	0.83	0.017	0.019	25	6.0	0.02000	0.37	0.0340	0.0083	4.1
	23.5	0.83	0.015	0.015	34	7.1	0.01600	0.36	0.0270	0.0066	4.1
50 "	24.0	0.84	0.035	0.034	35	5.5	0.02800	0.41	0.0560	0.0115	5.0
	22.7	0.84	0.025	0.028	35	6.0	0.02500	0.40	0.0480	0.0095	5.0
	23.0	0.84	0.035	0.032	35	5.5	0.02500	0.40	0.0560	0.0114	5.0
60 "	23.5	0.89	0.040	0.043	27	6.5	0.04900	0.47	0.0730	0.0116	6.3
	25.6	0.89	0.030	0.035	24	6.3	0.03500	0.43	0.0600	0.0099	6.1
	25.6	0.90	0.035	0.043	24	5.2	0.03300	0.43	0.0730	0.0106	6.9
70 "	27.2	0.92	0.050	0.050	17	6.8	0.08000	0.53	0.0840	0.0104	8.0
	26.7	0.90	0.040	0.038	17	6.8	0.06600	0.51	0.0650	0.0095	6.9
	23.5	0.91	0.045	0.040	17	4.6	0.05700	0.49	0.0680	0.0095	7.2
80 "	24.0	0.93	0.065	0.074	18	5.6	0.10300	0.57	0.1230	0.0133	9.2
	23.2	0.92	0.060	0.060	17	5.5	0.09100	0.55	0.1000	0.0120	8.3
	22.4	0.92	0.060	0.051	18	5.6	0.07700	0.53	0.0860	0.0107	8.0
85 "	23.0	0.93	0.055	0.070	12	6.4	0.17000	0.65	0.1170	0.0124	9.4
	25.6	0.93	0.052	0.063	12	7.0	0.16000	0.63	0.1050	0.0117	9.0
	21.4	0.93	0.050	0.055	12	6.0	0.14000	0.61	0.0920	0.0102	9.0
90 "	22.7	0.93		0.090	12	5.2	0.18500	0.66	0.1500	0.0165	9.1
	24.8	0.93		0.083	17	7.3	0.15000	0.63	0.1380	0.0147	9.3
	24.0	0.93	0.080	0.080	17	6.0	0.12600	0.60	0.1340	0.0146	9.3

which were dialyzed against a buffered phosphate solution, showed the same phenomenon, though here the columns raised did not exceed 1 to 2 cm. during the time of the experiment.

Fig. 2 shows in detail the diffusion of methemoglobin through the same six grades of membranes. It will be observed that diffusion through the 40, 50, 60, and 70 alcohol membranes is more rapid than with Congo red; for the 85 alcohol membranes,

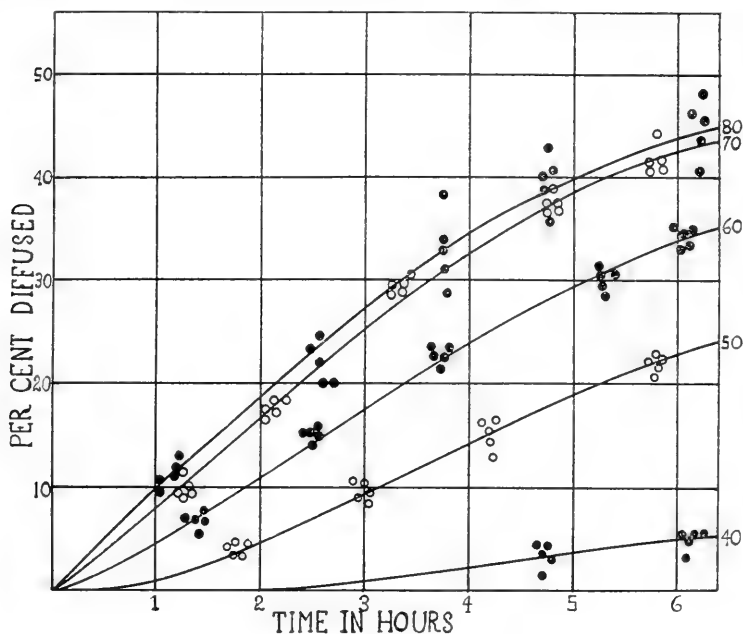


FIG 2. Diffusion of methemoglobin at 35°C. The numbers at the margin are the alcohol numbers of the membranes. The 85 alcohol series is omitted as the points have the same distribution as for the 80 alcohol series.

it is slower. Also, there is no essential difference in the diffusion of this substance through the 70, 80, and 85 membranes. It is probable that the methemoglobin molecule is smaller than that of Congo red, hence it passes more easily through the less permeable membranes. Further increase in permeability beyond the 70 alcohol membrane can no longer hasten the diffusion, which is limited by the diffusion constant of methemoglobin in water.

Congo red, with a larger aggregate, is more retarded by the lower membranes of the series; but being highly ionized relative to the methemoglobin, its diffusion constant in water is greater and it passes the 85 alcohol membranes more rapidly than the latter substance.

The curves for oxyhemoglobin and carboxyhemoglobin are not given, as they so closely resemble those of methemoglobin.

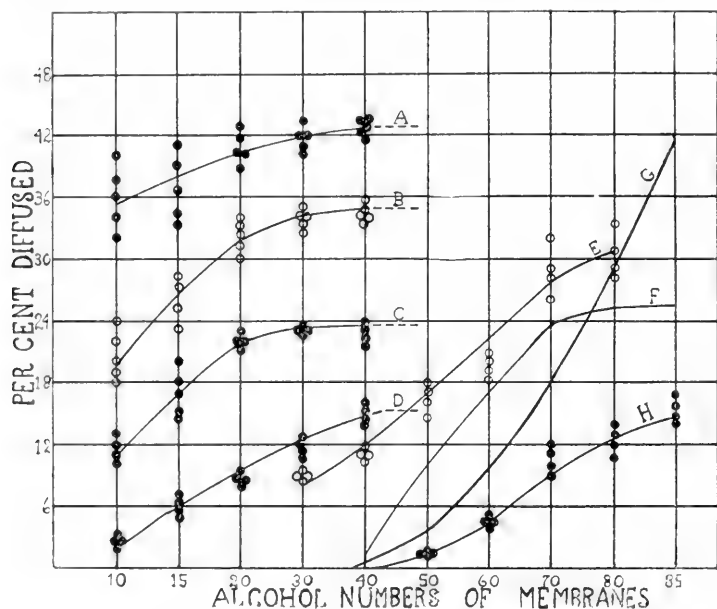


FIG. 3. A, sodium chloride, 10 minutes at 20°C. B, potassium dihydrogen phosphate, 10 minutes at 20°C. C, saccharose, 30 minutes at 20°C. D, indigo carmine, 20 minutes at 20°C. E, primary proteose, 2½ hours at 20°C. F, methemoglobin, 3 hours at 35°C. G, Congo red, 3 hours at 35°C. H, dialyzed serum, 3 hours at 35°C.

The behavior of indigo carmine and safranin was also studied in detail. Indigo carmine diffuses very slowly through the 10 alcohol membranes; the speed of diffusion increases with the alcohol numbers up to the 40 alcohol membranes, after which there is no further appreciable increase. The behavior of this substance is given in abbreviated form in Fig. 3. Safranin is somewhat less diffusible than indigo carmine; otherwise their curves are very similar.

Test substances, not colored, were tested for one time interval only. The behavior of these substances is shown in Fig. 3. Here the alcohol numbers of the membranes are plotted against the percentage of test substance diffused; the times and temperatures are indicated. Congo red and methemoglobin are included for comparison. The points on these two curves are omitted for the sake of simplifying the figure, as they may be found in Figs. 1 and 2. The curves of oxyhemoglobin and carboxyhemoglobin, safranin, and raffinose are also omitted for the same reason. With all the test substances, it is apparent that the permeability of the membranes increases with their alcohol numbers.

It is not altogether certain that the rise in the sodium chloride curve represents a real rise in the permeability of the membrane series to this test substance. If we consider a membrane as made up of a solid meshwork containing water-filled pores or spaces, then the true diffusing area is not the area of the membrane, but the sum of the areas of the pores. The proportion of water-filled spaces is not the same throughout the membrane series, as may be seen by referring to Column 3 of Table II. The 20 alcohol membranes have, on this theory, one-sixth more water-filled space than the 10 alcohol membranes of the same area. This is sufficient to account for the rise of the sodium chloride curve. This line of reasoning will not, however, explain away the rise in the curves of the other test substances studied as over 50 per cent more of phosphate, and 100 per cent more of saccharose diffused through the 20 alcohol membrane than through the 10, in the same length of time. The 70 alcohol membranes have about one-twelfth more water-filled space than the 50 alcohol membranes, yet twenty times as much serum protein diffused through them in the same length of time.

Fig. 4 shows the effect of different temperatures on the rate of diffusion of methemoglobin through 70 alcohol membranes. At the lower temperatures it will be noticed that the rise in the curve is preceded by an appreciable flat portion, representing a period during which little or no test substance diffuses through. The membrane, apparently, must be "saturated" with the substance before any of it can appear in the dialysate. If a methemoglobin solution is filtered under low pressure, through a 60

or 70 alcohol membrane, the first few cubic centimeters are colorless; only after the membrane is saturated with the test substance will any of it come through. If a very dilute solution, such as 1 to 1,000 Congo red, is used in diffusion experiments, little or none may appear in the dialysate even when the membrane is very permeable to that substance. To obtain consistent results with Congo red, it was found necessary to use a more

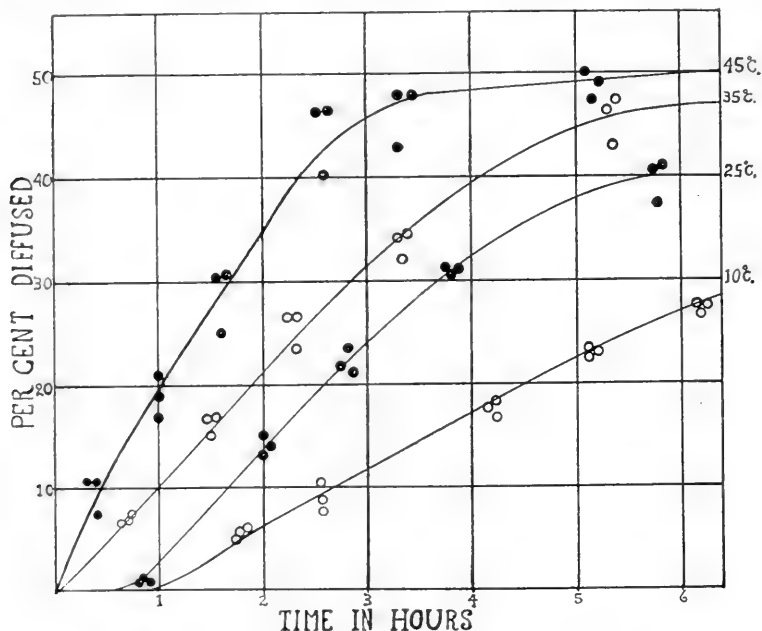


FIG. 4. Diffusion of methemoglobin through 70 alcohol membranes at different temperatures.

concentrated solution, so that the amount taken up by the membrane is small in comparison to the total amount.

The velocity of the filtration of water through membranes was used by Bechhold (1908), not only for demonstrating differences of permeability but also for calculating the diameters of the pores. This method is based upon the application to membranes of the law of Poiseuille for the passage of fluid through a capillary. The membrane is considered as a number of capillaries whose length is the thickness of the membrane.

This law states that  $Q = k \frac{PD^4}{L}$ ; where  $Q$  is the quantity of fluid passed through unit area in unit time,  $P$  the pressure,  $D$  the diameter of the capillary (pore),  $L$  the length of the capillary (thickness of membrane), and  $k$  a constant depending on the nature of the fluid, the temperature, and the units chosen. From this equation, it follows that  $D = \sqrt[4]{\frac{QL}{kp}}$ . Hence, with

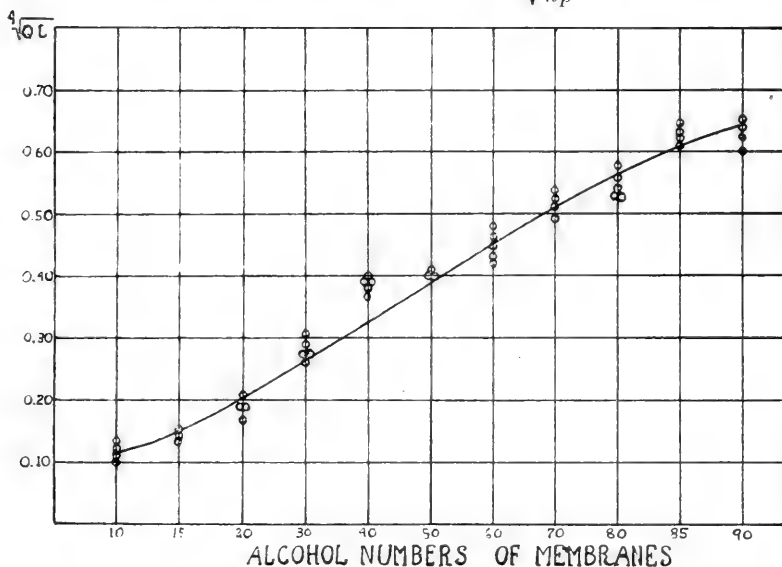


FIG. 5. Values of  $\sqrt[4]{QL}$  for the membrane series. Pressure, 60 mm. of Hg; temperature, 20°C.

constant pressure and temperature,  $D$  will vary as  $\sqrt[4]{QL}$ . The value of  $QL$  can be readily calculated from the experimental data. This has been done in Table II. Only three membranes of each series are given in this table, selected to show maximum, mean, and minimum values of  $QL$ . The values of  $\sqrt[4]{QL}$  are plotted in Fig. 5. No attempt was made to determine the value of the constant  $k$ , without which  $D$  cannot be computed.

#### *Method of Conducting Water Filtration Experiments.*

Bulbs of about 10 cc. capacity were blown from 8 mm. tubing; the two ends were cut down to 2 cm. in length. Each membrane

was attached to one of the ends of a bulb with a strong rubber band; the joint was dried with a towel and painted over with a thick 30 alcohol solution of collodion. When dry, the joint was tested for air-tightness. The sac was emptied and 12 cc. of distilled water were measured into it. A rubber tube over the other end of the bulb, led to a pressure tank and manometer. The filled sac was completely immersed in a test-tube of water, which stood in a constant temperature bath. A pressure of 60 mm. of Hg and a temperature of 20°C. were used in all experiments. Filtration was continued until the bulb, but not the sac, was empty. After filtration, the sac was emptied and tested for leaks; the volume of the residual water was measured.

The thickness of the membranes was measured with a micrometer, taking the mean of several readings at different places. The thickened bottom of the sac was removed; a piece 6 cm. long was cut off, rapidly blotted between filter papers, and weighed in a weighing bottle. This gave the wet weight. Each membrane was then air-dried and weighed again. The specific gravity of dry parlodion was found to be 1.608 at 20°C.

The volume in cubic centimeters occupied by the membrane is then equal to

$$\text{Wet weight} - \text{dry weight} + \frac{\text{dry weight}}{1.608}$$

all weights being expressed in grams. Dividing this value by the area of the membrane gives its thickness (Column 5, Table II). As the thickness obtained in this manner gave more consistent results than those obtained by measuring with the micrometer, this was used in calculating the value of  $QL$ . In making this calculation, the unit for  $L$  was taken as 0.01 mm.

The ratio of water-filled space to total volume was calculated as follows:

$$r = \frac{\text{wet weight} - \text{dry weight}}{\text{volume}}$$

Walpole (1915) and Brown (1915, 1917) showed that the ratio of the dry to the wet weight of a membrane was an index of its permeability. Their conclusion is fully supported by this investigation, as may be seen in the last column of Table II.



In the course of this investigation, a number of other substances were added to the alcohol-ether solvents, in the hope of improving the series. The most promising were certain of the organic acids. If acetic acid, for instance, is added to the solvent, it becomes much easier to make the membrane; instead of shrinking to the glass on immersing in water, the collodion film loosens from it readily and slips off with ease. 10 to 20 per cent of acetic acid seems also to give added durability and elasticity to the resulting membrane, and it increases the amount of non-solvent, such as glycerol or water, that may be added to the solution. The addition of this amount of acetic acid makes the membranes less permeable (only the 50 and 70 alcohol membranes were tried). Oxalic and citric acids and phenol made 70 alcohol membranes slightly more permeable when added in 5 per cent amounts. Lactic acid, however, added to the solutions in amounts varying from 10 to 30 per cent, greatly increases the permeability of the resulting membranes. Glycerol and water both increase permeability, though neither are as effective as lactic acid.

Since the work of Metchnikoff, collodion sacs have been extensively used in bacteriology and serology. For this work, it is desirable to sterilize the membranes by heat. Heating is accompanied by two undesirable changes, marked shrinkage and marked decrease in permeability. This matter has recently been investigated by Gates (1921), who gels his membranes in 95 per cent alcohol before immersing them in water; on heat sterilization, his membranes shrink about 33 per cent in volume and are still readily permeable to simple salts and glucose, though quite impermeable to protein and hemoglobin. It would seem desirable to prepare membranes that would allow the passage of proteins after heat sterilization; with this in view, the following experiments were conducted:

Sacs were prepared from the 80 alcohol solution. Some were immersed in 95 per cent alcohol, after the method of Gates. Others were made from the 80 alcohol solution plus 10 per cent of lactic acid, and immersed directly into water and washed free from acid. When autoclaved at 20 pounds pressure for 30 minutes, both sets of membranes shrank over 50 per cent in volume; when sterilized by three steamings in the Arnold

sterilizer, the shrinkage was very nearly 33 per cent for both sets of membranes. When tested with methemoglobin solution at 35°C., both sets of membranes were found to transmit it readily, the lactic acid membranes being more permeable. The latter had the permeability of unheated 60 alcohol membranes. Sacs made by both methods are serviceable and strong; they will stand over 25 cm. Hg of pressure without bursting. *Staphylococcus aureus* was grown in such sterile membranes for 2 months without contaminating the surrounding broth. *Bacillus influenzae* was grown in pure culture in plain broth without hemoglobin by growing it on the inside of a sterilized sac, with a living culture of staphylococcus, streptococcus, or pneumococcus growing on the outside; controls without the symbiotic organisms showed no growth of *Bacillus influenzae*. By this means it is possible to study the symbiosis of organisms while keeping each of them in pure culture.

The value of a method depends, among other things, on whether the results with it can regularly be reproduced. Most of the experiments described above were repeated many times, in whole or in part; fresh collodion solutions were made up several times from different lots of materials. Results obtained with membranes in the series between the alcohol numbers of 20 and 85 could be reproduced without any trouble. Such variations as occurred between different batches are believed to have been due to the presence of small amounts of water in solution. With the extremes of the series, more difficulty was encountered. The 90 alcohol membranes gave such irregular results that they have not been included in the series; they were several times found to be less permeable than the 85 or even the 80 alcohol membranes. This may in part have been due to their great variations in thickness, as the viscosity of the 90 alcohol solutions was found to be very variable. By referring to Table I, it will be seen that very small quantities of moisture suffice to cause great changes in viscosity. Variations in permeability were also found in the 10 and 15 alcohol membranes of different batches. One batch gave an almost horizontal line for the diffusion of sodium chloride and potassium dihydrogen phosphate, when plotted out as in Fig. 3. Three other batches gave more constant results.

## SUMMARY.

A simple method of preparing a graded series of collodion membranes of a wide range of permeability is presented, with quantitative data on the diffusion of various test substances through the different grades of the series.

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# THE DIRECT QUANTITATIVE DETERMINATION OF SODIUM, POTASSIUM, CALCIUM, AND MAGNESIUM IN SMALL AMOUNTS OF BLOOD.

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(Received for publication, June 8, 1921.)

The concentration of sodium, potassium, calcium, and magnesium in the blood of animals has been determined by Bunge (1), Abderhalden (2), and more recently by Greenwald (3). These investigators used from 25 to 100 cc. of blood for their determinations. Such quantities of blood cannot conveniently be used in studies with patients, particularly children. We have therefore devised a method by means of which the concentration of all these elements may be quantitatively determined on 7 cc. of blood.

## *Principle of the Method.*

Deproteinization is carried out by means of the trichloroacetic acid method recommended by Greenwald (4). The determinations of the individual elements, except that of magnesium, are then made directly on separate aliquots of the deproteinized fluid by modifications of methods previously described by us for serum (5, 6, 7). The inorganic or acid-soluble phosphorus may also be determined by any of the well known micro methods (8) on a portion of the supernatant fluid corresponding to 0.5 or 1.00 cc. of blood.

## *Methods.*

*Collection of Material and Deproteinization.*—25 cc. of distilled water are placed in a 50 cc. volumetric flask which is then weighed. From 7 to 8½ cc. of blood are obtained by means of a 10 cc. graduated syringe and slowly added to the water in the flask. The flask should be continuously rotated during this procedure which

completely hemolyzes the blood. The flask and contents are again weighed and the exact amount of blood added thereby determined. 1 or 2 drops of octyl alcohol are added followed by 12 to 13 cc. of 12 per cent trichloroacetic acid which are added slowly while the flask is rotated. The contents are thoroughly mixed and allowed to stand 10 minutes. Water is added to 50 cc., the contents are again mixed, transferred to a large centrifuged tube and centrifuged for 5 to 10 minutes at about 1,000 revolutions per minute. The supernatant fluid is poured off and an aliquot (generally 35 cc.) is placed in a beaker and evaporated. The presence of a few particles of the precipitate which sometimes float on the surface of the supernatant fluid does not interfere with the subsequent determinations. If the particles are very numerous, the fluid may be allowed to stand in the ice chest for a few hours. They will then have settled to the bottom. The supernatant fluid may be kept at this stage for at least 2 weeks before completing the determination. After the aliquot has been evaporated to dryness the residue is dissolved in 0.1 N HCl and is transferred to a volumetric flask and the volume made up to 10 cc. This fluid has the appearance of serum. Should it be cloudy it may be centrifuged for a few minutes when a clear, straw-colored supernatant liquid will be obtained. The sodium, potassium, and calcium determinations are done directly on this material while the magnesium determination is done on the first supernatant fluid obtained from the calcium determination.

#### *Sodium Method.*

4 cc. of the material prepared as outlined above are placed in a platinum dish and evaporated to about 2 cc. A drop of phenolsulfonephthalein is added and the contents are made just alkaline with 10 per cent KOH (generally about 10 to 12 drops will suffice). 10 cc. of the potassium pyroantimonate reagent are added followed by 3 cc. of 95 per cent alcohol. The alcohol should be added drop by drop and the specimen stirred with a rubber-tipped rod. After standing 30 minutes the precipitate is transferred to a weighed Gooch crucible and washed with 5 to 10 cc. of 30 per cent alcohol. The crucible is dried at 110° C. for 1 hour,<sup>1</sup> cooled in a desiccator for 30 minutes, and weighed.

<sup>1</sup> The temperature should be gradually raised to 110°C.

The weight of the precipitate divided by 11.08 equals the number of mg. of sodium present in the sample.

The method of preparation of the potassium pyroantimonate reagent has been fully described in a former paper on the determination of sodium in serum (5). The details of the method of preparation of the Gooch crucibles and the precautions to be observed during the addition of the alcohol and the filtration, and also the care of the platinum are fully outlined in the same paper.

For the determination of sodium in solutions of blood ash we used the same procedure as described for the determination of this element in solutions of the ash of urine and stools (9).

#### *Potassium Method.*

0.2 cc. of the material prepared as previously outlined is placed in a graduated centrifuge tube. 0.5 cc. of water is added followed by 0.5 cc. of a solution of sodium nitrite prepared by dissolving 15 gm. of potassium-free sodium nitrite (Merek) in 30 cc. of water. The contents of the tube are thoroughly mixed and allowed to stand for 5 minutes.<sup>2</sup> Water is added to 4 cc. and the contents are again mixed. 2 cc. of the sodium cobalti-nitrite reagent are added drop by drop. The contents of the tube are mixed and allowed to stand for a half hour, then centrifuged for 7 minutes at about 1,300 revolutions per minute. The precipitate will then be found at the bottom of the tube. All but 0.2 to 0.3 cc. of the supernatant fluid is removed. This is accomplished by means of the following apparatus. Through one opening of a two-holed cork is inserted a glass tube by means of which a positive pressure can be made in the centrifuge tube. Through the other hole is a tube which reaches to about 3 or 4 mm. above the precipitate. The lower end of this tube is drawn out to a

<sup>2</sup> If the sodium nitrite is not added, it will be found that the precipitate obtained on the addition of the cobalti-nitrite reagent will float on the surface of the fluid and adhere to the sides of the tubes. The precipitate will also adhere to the sides unless the tubes have been previously cleaned with the use of a brush, washed out with a strong cleaning fluid (commercial  $\text{H}_2\text{SO}_4$  and dichromate) and then thoroughly rinsed with distilled water. Low results will be obtained unless these procedures are carried out.

bore of about 1 mm. and curved so that the opening is directed upward. By fitting the cork to the centrifuge tube and blowing through the first opening the supernatant fluid can be readily removed without disturbing the precipitate. 5 cc. of water are allowed to run down the side of the tube which is then gently agitated so that the added water is mixed thoroughly with the residual reagent. Care should be taken that the precipitate itself is disturbed as little as possible. This may be accomplished by holding the tube vertically and gently hitting the lower end with a circular motion. The brown fluid may be seen to rise and mix with the supernatant fluid. The tube is then centrifuged for 5 minutes. The procedure is repeated three times so that the precipitate is washed four times in all. The supernatant fluid from the last washing should be perfectly clear. After the removal of the fluid from the final washing the precipitate is ready to be titrated.

*Titration.*—An excess of 0.02  $\times$  potassium permanganate is added (1.6 to 2 cc. are sufficient for normal blood), followed by 1 cc. of approximately 4  $\times$  sulfuric acid. The precipitate is then thoroughly mixed with the fluid by means of a glass rod. The sample is heated in the boiling water bath for 45 to 60 seconds at the end of which time the solution should be clear and still pink. If all the precipitate is not oxidized, the contents will be cloudy and the intensity of the color will be seen to diminish. Heating should then be continued until the solution is clear but still pink. When the heating is continued too long, the contents again become cloudy and have a brownish color. If this is allowed to happen, the sample must be discarded as high results will be obtained. An amount of 0.01  $\times$  sodium oxalate sufficient to decolorize the solution completely (generally 2 cc.) is promptly added. The excess of oxalate is then determined by titrating to a definite pink color with 0.02  $\times$  potassium permanganate delivered from a micro-burette graduated in 0.02 cc.

The details for the calculation of the amount of potassium present in the sample and also the methods for the preparation of the reagents are given in a former paper (9).

For the determination of potassium in solutions of blood ash we placed a quantity of fluid equal to 0.1 or 0.2 cc. of blood in a graduated centrifuge tube, added water to 2 cc., and then



slowly added 1 cc. of the cobalti-nitrite reagent. The subsequent steps were the same as described for the determination of this element in serum (5).

*Calcium Method.*

4 cc. of the material prepared as outlined above are placed in a graduated centrifuge tube previously cleaned with commercial  $\text{H}_2\text{SO}_4$  and potassium dichromate. 1 cc. of saturated ammonium oxalate is added, followed by 2 cc. of a filtered saturated solution of sodium acetate. The contents are mixed and allowed to stand for 1 hour. The volume is made up to 8 cc. with distilled water, mixed, and then centrifuged for 15 minutes at about 1,300 revolutions per minute. This throws all the calcium oxalate precipitate to the bottom of the tube. All but 0.3 cc. of the supernatant fluid is removed by means of the apparatus described under the potassium method. The remaining fluid and the precipitate are mixed by tapping the tube. Enough 2 per cent ammonia (2 cc. of concentrated ammonia diluted to 100 cc.) is then added to bring the volume to 4 cc., care being taken to wash the sides of the centrifuge tube free from adherent oxalic acid. The tube is then centrifuged for 5 minutes. This procedure is repeated twice, thus making three washings in all. After the third washing the supernatant fluid is removed, the tube is shaken to suspend the precipitate, 2 cc. of approximately  $\text{N}$  sulfuric acid are added and the tube is warmed in the boiling water bath for a few minutes and titrated with 0.01  $\text{N}$  potassium permanganate until a definite pink color persists for at least 1 minute when viewed under a good light against a white background. The strength of the permanganate solution is determined by titrating against a 0.01  $\text{N}$  sodium oxalate (Sörensen).

The details for the calculation of the amount of calcium present in the sample and also the methods for the preparation of the reagents are given in a former paper (9).

For the determination of calcium on 0.1  $\text{N}$   $\text{HCl}$  solutions of blood ash the procedure is identical with that described above. The blood ash solution was made up so that 1 cc. corresponded to 1 cc. of blood. It was allowed to stand for 2 or 3 weeks to allow the sediment to settle. 2 cc. of this solution were used for each determination.

*The Magnesium Method.*

5 cc. of the supernatant fluid from the calcium determination are measured into a 30 cc. beaker, 1 cc. of  $(\text{NH}_4)_2\text{HPO}_4$  solution is added and then 2 cc. of concentrated ammonia. The next day the sample is filtered through a well packed Gooch crucible, washed ten times with 5 cc. of 10 parts of concentrated ammonia to 90 parts of water, then twice with 95 per cent alcohol made alkaline with ammonia. The crucible is returned to the beaker and dried for a few minutes at  $80^\circ \text{C}$ . in the oven.

10 cc. of  $0.01 \text{ N HCl}$  are added to the crucible and after a few hours the entire material is transferred to a test-tube, centrifuged, and 5 cc. of the supernatant fluid are measured into a flat bottomed colorimeter tube graduated for 10 cc., which contains 2 cc. of the iron thiocyanate solution. The volume is then made up to 10 cc. with  $0.01 \text{ N HCl}$ , a rubber stopper inserted, and the fluid mixed. A series of standards is prepared by adding varying amounts of a known  $\text{NH}_4\text{MgPO}_4$  solution in  $0.01 \text{ N HCl}$ , to 2 cc. samples of thiocyanate solution and bringing the volume up to 10 cc. as in the unknown samples. The color is compared by looking through the entire length of the liquid column against a white background.

*Calculation.*—The calculation is the same as in the original method:  $\frac{\text{Reading (cc. of standard solution)} \times 0.01 \times 2 \times 8/5 \times 100}{\text{cc. blood used in Ca determination}} = \text{mg. of magnesium in 100 cc. of blood.}$

*Preparations of Reagents.*

1. *Ammonium Magnesium Phosphate Standard.*—This solution is made by dissolving 0.102 gm. of air-dried magnesium ammonium phosphate ( $\text{MgNH}_4\text{PO}_4 \cdot 6\text{H}_2\text{O}$ ) in 100 cc. of  $0.1 \text{ N}$  hydrochloric acid and diluting to 1 liter with water. Of this solution 1 cc. is equivalent to 0.01 mg. of magnesium. Magnesium ammonium phosphate loses water of crystallization when heated and must therefore be dried at room temperature. Commercial preparations of the salt are generally unreliable; it should be prepared by precipitation of pure solutions (10).

2. *Ammonium Phosphate Solution*.—Ammonium phosphate solution is made as follows: 25 gm. of  $(\text{NH}_4)_2\text{HPO}_4$  are dissolved in 250 cc. of  $\text{H}_2\text{O}$ . 25 cc. of concentrated ammonia are added and the mixture is allowed to stand over night. The following day it is filtered, the filtrate is boiled to remove the excess of ammonia, cooled, and made up to 250 cc. This solution is diluted five times with water.

3. *The Ferric Thiocyanate Solution*.—This solution is made from two solutions which are mixed an hour before use. Solution A is 0.3 per cent ammonium thiocyanate. Solution B is 0.3 per cent ferric chloride, made up from the salt with its contained water of crystallization, adding a few drops of acid, if necessary, to clear the solution. 5 cc. portions of Solutions A and B are mixed and the whole is diluted to 40 cc. with water.

4. *10 Per Cent Ammonia*.—100 cc. of concentrated ammonia are diluted to 1 liter.

#### *Protocols.*

We have previously shown (5) that sodium may be precipitated quantitatively, as sodium pyroantimonate, from solutions of blood salts. Since the composition of the supernatant fluid after deproteinizing blood with trichloroacetic acid is comparable, except for the small amount of residual protein and the non-protein nitrogenous constituents, to some of the solutions of blood salts which we have analyzed, we have considered it unnecessary to repeat this demonstration here.

Table I shows that the sodium determinations when performed on the deproteinized solutions yield results practically identical with those obtained on a solution of the whole blood ash. The absolute values vary from 170 to 225 mg. of sodium per 100 cc. of blood.

Table II. The concentration of potassium seems to vary considerably in the blood of different animals. Bunge found 213 mg. of potassium in 100 cc. of pig's blood, 227 mg. in that of the horse, but only 34 mg. in the same volume of cow's blood and 20 mg. per 100 cc. of dog's blood. The lowest figure which Abderhalden reports is 21 mg. of potassium per 100 cc. of dog's blood while the highest figure is 227 mg. per 100 cc. of horse's blood. We have found that the potassium content of human

blood varies from 153 to 202 mg. of potassium per 100 cc. It varies with the percentage of corpuscles. It might be mentioned that the cobalti-nitrite reagent gives no precipitate when added to ferric chloride or trichloroacetic acid. We have shown elsewhere that none of the constituents of serum except potassium

TABLE I.  
*Sodium Determinations on Blood.*

Sample.	Plasma.	Na per 100 cc. of blood ashed.	Na per 100 cc. of blood deproteinized.
	<i>per cent</i>	<i>mg.</i>	<i>mg.</i>
1	65	225	216
2	55	170	175
3	65	207	207
4	58	187	186
5	56	185	193
6	60	198	200
Average.....	60	195	196

TABLE II.  
*Potassium Determinations on Blood.*

Determinations on ashed blood.			Determinations on blood treated with trichloroacetic acid.		
Sample.	Plasma.	K per 100 cc. of blood.	Sample.	Plasma.	K per 100 cc. of blood.
		<i>mg.</i>			<i>mg.</i>
1	61	172	7	61	180
2	60	187	8	62	175
3	57	188	9	57	202
4	68	153	10	59	193
5	58	186	11	65	164
6	57	200	12	65	169
			13	56	201
Average...	60	181		61	183

yields demonstrable amounts of insoluble nitrites with this reagent (6).

Table III. The concentration of calcium in serum or plasma is singularly constant (7). On the other hand the concentration of this element in blood varies inversely as the corpuscular con-

tent. The results which we obtained varied from 5.3 to 6.8 mg. of calcium per 100 cc. of blood. The individuals from whom the samples were obtained were all normal adults. We have found that the addition of ferric chloride to a solution of blood salts

TABLE III.  
*Calcium Determinations on Blood.*

Determinations on ashed blood.			Determinations on blood treated with trichloroacetic acid.		
Sample.	Plasma.	Ca per 100 cc. of blood.	Sample.	Plasma.	Ca per 100 cc of blood.
	<i>per cent</i>	<i>mg.</i>		<i>per cent</i>	<i>mg.</i>
1	58	5.3	8		6.3
2	57	5.3	9		5.3
3	72	6.7	10		6.1
4	59	6.2	11		5.3
5	58	5.3	12		5.7
6	65	5.9	13		6.4
7	57	5.5			
Average...		5.7			5.8

TABLE IV.  
*Magnesium Determinations on Blood.*

Determinations on ashed blood.		Determinations on blood treated with trichloroacetic acid.	
Sample.	Mg. per 100 cc. of blood.	Sample.	Mg. per 100 cc. of blood.
	<i>mg.</i>		<i>mg.</i>
1	2.8	5	2.6
2	2.8	6	4.0
3	3.8	7	3.8
4	2.3	8	3.8
Average.....	2.9		3.5

does not interfere with the quantitative determination of calcium. The results obtained on the deproteinized material and on the solutions of blood ash are practically identical.

Table IV. The concentration of magnesium in the blood of various animals has been found by Bunge and Abderhalden

to be fairly constant, varying only from 2 to 4 mg. per 100 cc. of blood (2). We have found that the concentration of this element in the blood of the adult male varies from 2.3 to 4.0 mg. per 100 cc.

#### CONCLUSIONS.

1. A method has been described by means of which sodium, potassium, calcium, and magnesium may be quantitatively determined on only 7 cc. of blood.

2. The basis of this method is deproteinization by means of trichloroacetic acid. The quantitative determination of each of these elements is then made on aliquots of the supernatant fluid by modifications of procedures recently described for serum.

3. The results obtained by these methods on deproteinized blood agree well with those obtained on solutions of blood ash.

4. We have found the concentration of these elements in 100 cc. of human blood to be as follows: sodium, 170 to 225 mg.; potassium, 153 to 201 mg.; calcium, 5.3 to 6.8 mg.; and magnesium, 2.3 to 4 mg.

5. The concentration of these elements in normal blood varies more than in normal serum. This is due to the variations in the corpuscular content of the blood.

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## PHOSPHORIC ESTERS OF SOME SUBSTITUTED GLUCOSEs AND THEIR RATE OF HYDROLYSIS.

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(Received for publication, July 7, 1921.)

The purpose of the preparation of the substance to be described in this communication was developed in a previous publication by Levene and Yamagawa. The interest, as was explained, is both of a theoretical and a practical nature. The question to be answered is the following. Does the stability of the inorganic radicle in a phosphoric ester of a sugar (free or substituted) depend on the position of the inorganic radicle?

The answer to this question is given in the following table of the constants of hydrolysis of three 1-2-acetone phosphoric acid glucoses.

	K
1-2-Monoacetone phosphoric acid glucose.....	44 ( $10^{-3}$ )
1-2-Monoacetone-6-phosphoric acid glucose.....	58 ( $10^{-3}$ )
1-2-Monoacetone-3- or 5-phosphoric acid glucose <sup>1</sup> .....	24 ( $10^{-3}$ )

Substance 1 was prepared from 1-2-acetone glucose, hence the position of the phosphoric acid in it may be in any position from 3 to 6 (inclusive). Substance 2 was prepared in the course of preparation of the derivative from 1-2-3-5-diacetone, hence it is in the main 1-2-diacetone-6-phosphoric acid glucose. The third substance was prepared from 1-2-3-5-diacetone-6-benzoyl glucose. This was converted into 1-2-acetone-6-benzoyl glucose, this further into 1-2-acetone-3- or 5-phosphoric acid-6-benzoyl glucose, and finally the latter into 1-2-acetone-3- or 5-phosphoric

<sup>1</sup> The second sentence of the last paragraph on p. 324, *J. Biol. Chem.*, xliii, should read: Whereas the fourth was obtained by the action of phosphorus oxychloride on monoacetone glucose, the fifth is formed as a by-product by the action of phosphorus oxychloride on diacetone glucose.

acid glucose. Thus in Substance 2 the phosphoric acid radicle is attached to carbon atom 6, and in Substance 3 in position 3 or 5. The differences in the rate of hydrolysis are far beyond the limits of error of the method. It is interesting to note that the rate of hydrolysis of Substance 3 is practically the same as found in the earlier experiments of Levene and Yamagawa on 1-2-acetone-6-benzoyl phosphoric acid. This similarity is easily understood, since the benzoyl group being in position 6 is very labile, hence, in the course of hydrolysis the benzoyl derivative is soon transformed into the monoacetone phosphoric acid glucose. In the same manner is explained the fact that 1-2-acetone-6-phosphoric acid glucose, and 1-2-3-5-diacetone-6-phosphoric acid hydrolyze with the same velocity.

The analytical data on the barium salts of phosphoric esters, here reported, call for a special note. They are not so satisfactory as is desired. However, it has been the experience of this laboratory that when barium salts of pure phosphoric esters are obtainable only in an amorphous form, the analytical results are often not perfect. Thus it was found practically impossible to obtain a perfect agreement between the found and required elementary composition of the amorphous barium salt prepared from the crystalline adenosinphosphoric acid.

The observations on the phosphoric esters of the 1-2-3-5-methyl glucose and of the 1-3-5-6-methyl glucose are in harmony with those on the esters of the 1-2-acetone glucose.

Thus the rate of hydrolysis of 1-2-3-5-methyl-6-phosphoric acid glucose proceeded normally, giving an average ratio,  $K = 44 (10^{-3})$ , which is not far removed from that of the acetone glucose ester having the inorganic radicle in position 6, where  $K = 56 (10^{-3})$ . On the other hand, the rate of hydrolysis of 1-3-5-6-methyl-2-phosphoric acid glucose proceeded abnormally. There was noted a rapid hydrolysis at the beginning which reached a maximum after about 6 hours, and the further progress was very slow. The explanation of this result may be the following. The 1-3-5-6-methyl glucose is apparently very difficult to prepare in pure form. The material employed for coupling with phosphoric acid may have contained as an impurity some trimethyl glucose with either carbon atom 1 or carbon atom 6 free. Thus the material derived from this product may be a mixture of two



phosphoric esters, one with an inorganic radicle in position 1 or 6, which is labile, and the other in position 2 which is very stable. The first is decomposed rapidly, whereas the second proceeds at the low rate indicated in the table.

The estimations of the rate of hydrolysis were carried out by Miss I. Weber.

#### EXPERIMENTAL.

##### *$\alpha$ -Methyl Glucosidophosphoric Acid.*

Portions of 10 gm. of dried methyl glucoside are dissolved in 50 cc. of warm, water-free pyridine. This solution is cooled to  $-20^{\circ}\text{C}$ . and to it is added, in small portions, a solution of 7.8 gm. (1 mol) phosphorus oxychloride in 20 cc. of pyridine, also cooled to  $-20^{\circ}\text{C}$ .

It is important that the pyridine as well as the methyl glucoside should be absolutely dry. For this purpose the substance is dried over phosphorus pentoxide at  $100^{\circ}\text{C}$ . under a pressure of about 1 mm. The pyridine is boiled with reflux over barium oxide for 5 hours and then distilled. The pyridine and phosphorus oxychloride should be well cooled before mixing, and if the pyridine is dry no pyridine hydrochloride will settle out even after standing for some time.

After the phosphorus oxychloride in pyridine has been added to the solution of the glucoside, the reaction mixture is allowed to remain at  $-20^{\circ}\text{C}$ . for several hours, during which time a considerable amount of pyridine hydrochloride separates. The reaction mixture is now diluted with 20 cc. of ice water and allowed to come to room temperature when it is further diluted by pouring it into 200 cc. of cold water. An excess of barium hydrate (100 gm.) is added and the pyridine removed by distillation under diminished pressure. The temperature of the bath should not be above  $+30^{\circ}\text{C}$ .

When all the pyridine has been removed the solution is made just acid to Congo red with sulfuric acid. The hydrochloric acid is now removed by the addition of 30 gm. of silver sulfate. After shaking this mixture for half an hour it is filtered and the silver removed from the filtrate by hydrogen sulfide and the latter removed by a current of air.

An excess of barium hydrate is added and carbon dioxide passed into the solution until the reaction is neutral to litmus. The solution is now filtered and concentrated under diminished pressure and low temperature. When the volume has been reduced to one half of the original, the solution is filtered and the filtrate concentrated to a small volume which is then poured into a large volume of absolute alcohol. For purification the precipitate is dissolved in a small quantity of water, filtered, and reprecipitated with alcohol. The yield was 9.0 gm.

For analysis the substance is dried under diminished pressure at the temperature of water vapor.

0.1042 gm. of substance gave on combustion 0.0866 gm. of  $\text{CO}_2$  and 0.0310 gm. of  $\text{H}_2\text{O}$ .

0.2757 gm. of substance gave 0.0746 gm. of  $\text{Mg}_2\text{P}_2\text{O}_7$ .

0.0982 " " " " 0.0485 " "  $\text{BaSO}_4$ .

$\text{C}_7\text{H}_{13}\text{O}_6\text{H}_2\text{PO}_3$ . Calculated. C 30.7, H 4.8, P 11.0.

Found (calculated Ba-free). C 31.8, H 4.68, P 10.6.

The optical rotation in water was found:

$$[\alpha]_D^{20} = \frac{+1.32^\circ \times 100}{2 \times 1.615} = +81.8^\circ$$

#### *1-2-3-5-Diacetone Glucose.*

Fischer's latest method for the preparation of diacetone from  $\beta$ -glucose<sup>2</sup> in a somewhat modified form was employed.

75 gm. of dried  $\beta$ -glucose, which is readily prepared by the method of Behrend, are placed in an ordinary glass-stoppered acid bottle with 1,500 cc. of dried acetone containing 1 per cent hydrochloric acid. The bottle was shaken for 24 hours at  $30^\circ\text{C}$ . The contents are now dark-colored and nearly all the  $\beta$ -glucose has dissolved. The solution is filtered and neutralized to litmus with sodium methylate, which is prepared by dissolving 4 gm. of sodium in 400 cc. of dry methyl alcohol. When neutral the acetone solutions assume a much lighter color.

The solution is again filtered and concentrated to dryness under diminished pressure. A solid cake is formed and repeatedly extracted with warm petroleum ether from which on cooling, diace-

<sup>2</sup> Fischer, E., and Rund, C., *Ber. chem. Ges.*, 1916, xlix, 93.

tone glucose crystallizes. The diacetone glucose is further purified by the method described by Fischer. 600 gm. of  $\beta$ -glucose under proper conditions yield 350 gm. of diacetone glucose.

Although some monoacetone is also obtained the yield of this substance is comparatively small.

*1-2-3-5-Diacetone-6-Phosphoric Acid Glucoside.*

The action of phosphorus oxychloride on diacetone glucose depending upon certain conditions yields either a diacetone glucose phosphoric acid, the barium salt of which is soluble in absolute alcohol, or a monoacetone phosphoric acid, the barium salt of which is insoluble in alcohol.

10 gm. of thoroughly dried diacetone glucose are dissolved in 50 cc. of dry pyridine and cooled to  $-35^{\circ}$ . This low temperature is readily obtained by using a mixture of  $\text{CaCl}_2 + 6\text{H}_2\text{O}$  and ice. To the sugar solution are added in small amounts 5.4 gm. phosphorus oxychloride in 20 cc. of dry pyridine, likewise cooled to  $-35^{\circ}$ . The mixture is now transferred to a bath of  $-10^{\circ}$  and kept therein for 2 hours. During this time crystals of pyridine hydrochloride have separated. The reaction mixture is again cooled to  $-35^{\circ}\text{C}$ . and about 20 cc. of moist pyridine, also cooled to  $-35^{\circ}\text{C}$ ., added in such small quantities that the temperature will not rise above  $-10^{\circ}$ . This is then followed by the addition of a cold solution of 10 cc. each of pyridine and water and finally a small cake of ice.

The reaction product is now allowed to come to room temperature. An excess of barium hydrate is added, the pyridine removed under diminished pressure, and the hydrochloric acid removed by shaking with silver sulfate, and further treated as previously described.

The barium salt of diacetone phosphoric acid is very soluble in alcohol and is not precipitated by ether or acetone. On allowing the alcoholic solution to evaporate to dryness the barium salt is obtained as a fine white powder which when dry is no longer hygroscopic. It does not reduce Fehling's solution until after hydrolysis with acid.

For analysis the substance was dried to constant weight under diminished pressure at the temperature of water vapor.

0.1133 gm. of substance gave on combustion 0.1331 gm. of  $\text{CO}_2$  and 0.0482 gm. of  $\text{H}_2\text{O}$ .

0.0923 gm. of substance gave 0.0341 gm. of  $\text{BaSO}_4$ .

0.2770 " " " 0.0625 " "  $\text{Mg}_2\text{P}_2\text{O}_7$ .

$\text{C}_{12}\text{H}_{19}\text{O}_6\text{H}_2\text{PO}_3$ . Calculated. C 42.25, H 6.18, P 9.10.

Found (calculated Ba-free). C 41.5, H 6.18, P 8.90.

The rotation in water was found:

$$[\alpha]_D^{20} = \frac{-11^\circ \times 100}{1 \times 4.472} = -2.48^\circ$$

*1-2-Monoacetone Phosphoric Acid Glucoside from Diacetone Glucose.*

This substance is obtained by the action of phosphorus oxychloride on diacetone glucose when the temperature of the reaction mixture is allowed to rise above  $+10^\circ$  during the process of destroying the unutilized phosphorus oxychloride.

10 gm. of dried diacetone glucose are dissolved in 50 cc. of dry pyridine and cooled to  $-20^\circ$ . To this is added 5.4 gm. of phosphorus oxychloride dissolved in 20 cc. of pyridine also cooled to  $-20^\circ$ . The mixture is allowed to remain at  $-20^\circ$  for several hours and then 20 cc. of ice cold water are added. The temperature of the mixture rises to about  $+30^\circ$ . After allowing it to stand at room temperature for a while it is further diluted with water. An excess of barium hydrate is added and the pyridine removed by distillation under diminished pressure.

The product is treated with silver sulfate and barium hydrate as previously described. The final residue is soluble in water which on the addition of alcohol forms a gelatinous mass. The barium salt is precipitated from its aqueous solution by pouring it into a large volume of dry acetone. The salt is obtained as a fine white powder. It does not reduce Fehling's solution until after hydrolysis with acid.

For analysis the substance was dried under diminished pressure at the temperature of water vapor.

0.1032 gm. of substance gave on combustion 0.1016 gm. of  $\text{CO}_2$  and 0.0394 gm. of  $\text{H}_2\text{O}$ .

0.2679 gm. of substance gave 0.0653 gm. of  $\text{Mg}_2\text{P}_2\text{O}_7$ .

0.0893 " " " 0.0424 " "  $\text{BaSO}_4$ .

$\text{C}_9\text{H}_{15}\text{O}_6\text{H}_2\text{PO}_3$ . Calculated. C 35.75, H 6.00, P 10.28.

Found (calculated Ba-free). C 35.9, H 5.61, P 9.30.

The optical rotation in water was:

$$[\alpha]_D^{20} = \frac{0.35^\circ \times 100}{1 \times 5.116} = +6.8^\circ$$

*1-2-Monoacetone Phosphoric Acid Glucoside from Monoacetone Glucose.*

10 gm. of dry monoacetone glucose are dissolved in 50 cc. of dry pyridine and cooled to  $-30^\circ$ . To this is added 4.4 gm. of phosphorus oxychloride dissolved in 20 cc. of dry pyridine. The reaction mixture is allowed to stand for some time at  $-20^\circ$  and treated as described in the previous preparations. The residue is soluble in 95 per cent alcohol which on pouring into a large excess of dry ether precipitates the barium salt of monoacetone phosphoric acid glucoside. The yield was 9 gm.

The substance is dried for analysis under diminished pressure at the temperature of xylene vapor.

0.1103 gm. of substance gave on combustion 0.1056 gm. of  $\text{CO}_2$  and 0.0400 gm. of  $\text{H}_2\text{O}$ .

0.2940 gm. of substance gave 0.0744 gm. of  $\text{Mg}_2\text{P}_2\text{O}_7$ .

0.0980 " " " " 0.0438 " "  $\text{BaSO}_4$ .

$\text{C}_9\text{H}_{16}\text{O}_6\text{H}_2\text{PO}_3$ . Calculated. C 35.75, H 6.00, P 10.28.

Found (calculated Ba-free). C 35.4, H 5.42, P 9.55.

The optical rotation found was:

$$[\alpha]_D^{20} = \frac{+20^\circ \times 100}{1 \times 4.000} = +5.0^\circ$$

*1-2-Monoacetone-6-Benzoyl Phosphoric Acid Glucoside.*

The benzoyl monoacetone glucose was prepared from benzoyl diacetone glucose according to the method of Fischer.

10 gm. of dried benzoyl monoacetone glucose are dissolved in 50 cc. of dry pyridine and cooled to  $-30^\circ$ . To this are added in small amounts 4.7 gm. of phosphorus oxychloride dissolved in 20 cc. of dry pyridine, also cooled to  $-30^\circ$ . The mixture is allowed to stand at  $-30^\circ$  for several hours during which time crystals of pyridine hydrochloride have separated. Moist pyridine, cooled to  $-20^\circ$ , is now added, followed by a cake of ice. The mixture is allowed to come to room temperature, diluted with water,

and treated as previously described. The residue is soluble in absolute alcohol from which the barium salt is precipitated by pouring it into a large volume of dry ether. The yield was 10 gm.

For analysis the substance was dried under diminished pressure at the temperature of chloroform vapor.

0.1097 gm. of substance gave on combustion 0.1316 gm. of  $\text{CO}_2$  and 0.0426 gm. of  $\text{H}_2\text{O}$ .

0.2941 gm. of substance gave 0.0642 gm. of  $\text{Mg}_2\text{P}_2\text{O}_7$ .

0.0980 " " " " 0.0394 " "  $\text{BaSO}_4$ .

$\text{C}_{16}\text{H}_{20}\text{O}_7\text{H}_2\text{PO}_3$ . Calculated. C 47.4, H 4.95, P 7.64.

Found (calculated Ba-free). C 42.9, H 5.3, P 8.06.

The optical rotation was found:

$$[\alpha]_D^{25} = \frac{+0.26^\circ \times 100}{1 \times 2.184} = +11.94^\circ$$

From the analysis of the substance and from the fact that it is non-reducing it was supposed that in the process of preparation a small part of the benzoic acid radicle was removed. This assumption was corroborated by the estimation of the benzoic acid on hydrolysis of the substance.

1.000 gm. of barium salt was hydrolyzed with dilute sulfuric acid. The solution was extracted with ether, the ethereal solution dried with anhydrous sodium sulfate and concentrated. The residue consisted of benzoic acid and weighed 0.1376 gm. or 13.76 per cent. Theory required 22.6 per cent.

*1-2-Monoacetone Phosphoric Acid Glucose from  
1-2-Monoacetone-6-Benzoyl Phosphoric Acid Glucose.*

15 gm. of the barium salt of the phosphorated sugar were dissolved in 150 cc. of water and made just acid to Congo red with sulfuric acid. An additional 10 cc. of 2 N sulfuric acid were added and all made up to 200 cc. This was heated in a water bath at  $50^\circ\text{C}$ . for 70 minutes. The solution was cooled, filtered, and extracted with ether. The aqueous solution was made alkaline with barium hydrate and filtered from the barium phosphate. Carbon dioxide was passed until the reaction was neutral to litmus. The solution was again filtered and concentrated under diminished pressure at  $30^\circ\text{C}$ . The residue was soluble in 95 per

cent alcohol and the barium salt precipitated by pouring it into a large volume of dry ether. The yield was 6 gm.

For analysis the substance was dried under diminished pressure at the temperature of water vapor.

0.1071 gm. of substance gave on combustion 0.0979 gm. of  $\text{CO}_2$  and 0.0420 gm. of  $\text{H}_2\text{O}$ .

0.2811 gm. of substance gave 0.0658 gm. of  $\text{Mg}_2\text{P}_2\text{O}_7$ .

0.0937 " " " " 0.0446 " "  $\text{BaSO}_4$

$\text{C}_9\text{H}_{16}\text{O}_6\text{H}_2\text{PO}_3$ . Calculated. C 35.75, H 6.00, P 10.28.

Found (calculated Ba-free). C 35.67, H 6.24, P 9.48.

The substance does not reduce Fehling's solution until after hydrolysis with dilute acid.

The optical rotation found was:

$$[\alpha]_D^{20} = \frac{0.25^\circ \times 100}{1 \times 0.4000} = +6.25^\circ$$

#### *2-3-5-Trimethyl-Methyl Glucoside.*

The trimethyl glucoside was prepared by the method of Haworth.<sup>3</sup> Methyl glucoside was methylated in portions of 20 gm. each. After 300 gm. of glucoside had been methylated the combined product consisting of a mixture of the methylated glucosides was subjected to a fractional distillation. The progress of fractionation was controlled by means of the index of refraction as well as by methoxy determinations.

The product was separated into the following fractions:

Fraction.	<i>n</i>	$N_D^{20}$	$\text{CH}_3\text{O}$ <i>per cent</i>	Boiling point P = 0.15 mm. °C.
I	46°56.5'	1.4449	60.95	85
II	46°51.5'	1.4450	61.08	90
III	46°41.5'	1.4446	60.00	95
IV	46°01.5'	1.4502	56.67	105
V	45°11.5'	1.4550	52.30	110

The theory for tetramethyl-methyl glucoside requires 61.75 per cent  $\text{CH}_3\text{O}$  and trimethyl-methyl glucoside calls for 52.54

<sup>3</sup> Haworth, W. N., *J. Chem. Soc.*, 1915, cvii, 8.

per cent  $\text{CH}_3\text{O}$ . Fractions IV and V were again distilled, the main fraction boiling at  $108^\circ\text{C}$ .,  $P = 0.07$  mm.,  $n = 44^\circ 43'$ ,  $N_D^{20} = 1.45786$ ,  $D_4^{20} = 1.1477$ . Found  $M_p = 56.06$ .

Theory requires 55.917. The yield of this fraction was 137 gm. Methoxy determination:

0.1803 gm. of substance gave 0.7145 AgI (factor = 13.2) = 52.30 per cent of  $\text{CH}_3\text{O}$ . Theory requires 52.54 per cent.

*2-3-5-Trimethyl-6-Phosphoric Acid Methyl Glucoside.*

Portions of 10 gm. of the glucoside are dissolved in 50 cc. of dry pyridine and cooled to  $-30^\circ$ . To this mixture is added a solution of 6.5 gm. of phosphorus oxychloride in 20 cc. of pyridine, also cooled to  $-30^\circ\text{C}$ .

The reaction is rather feeble and only after some time pyridine hydrochloride begins to separate. The reaction mixture is allowed to stand for several hours at  $-20^\circ$  and then 20 cc. of cold moist pyridine are added. The product is now allowed to come to room temperature and further diluted with ice cold water.

After making alkaline with 60 gm. of solid barium hydrate, the pyridine is removed by distillation under diminished pressure. The reaction product is then treated with silver sulfate and barium hydrate.

The barium salt of this substance is very soluble in alcohol and acetone. It was purified by dissolving it in a small quantity of acetone and precipitating it in a large volume of dry ether. The salt is obtained as a white hygroscopic powder. For analysis it is dried under diminished pressure at the temperature of xylene vapor.

0.1122 gm. of substance gave on combustion 0.0462 gm. of  $\text{H}_2\text{O}$  and 0.1164 gm. of  $\text{CO}_2$ .

0.0908 gm. of substance gave 0.0382 gm. of  $\text{BaSO}_4$ .

0.2723 " " " " 0.0705 " "  $\text{Mg}_2\text{P}_2\text{O}_7$ .

$\text{C}_{10}\text{H}_{19}\text{O}_6\text{H}_2\text{PO}_3$ . Calculated. C 37.97, H 6.68, P 9.80.

Found (calculated Ba-free). C 37.50, H 6.12, P 9.45.

The rotation in water was found:

$$[\alpha]_D^{20} = \frac{-2.36^\circ \times 100}{1 \times 3.062} = +77.07^\circ$$



*3-5-6-Trimethyl-1-2-Acetone Glucose.*

3-5-6-Trimethyl-methyl glucoside was prepared by methylating 1-2-acetone glucose and converting the product into the glucoside. The methylation of monoacetone glucose had been attempted by Irvine and Scott<sup>4</sup> using the silver oxide and methyl iodide method. The results by this method are not satisfactory. Attempts to methylate the trimethyl glucose obtained after cleaving off the acetone were also unsuccessful. The following method was finally adopted.

Portions of 25 gm. of monoacetone glucose were methylated with an excess of dimethyl sulfate by the method of Haworth. The temperature of the bath should not exceed 55°; the methylation should proceed rather slowly, and the alkali should be always present in a slight excess. To 25 gm. of monoacetone there were used 150 cc. of 30 per cent sodium hydroxide and 90 cc. of freshly distilled dimethyl sulfate. After all the reagents had been added the stirring was continued for one hour at the same temperature. The solution was then cooled and ammonium hydroxide added to destroy any unutilized dimethyl sulfate. The solution was extracted with chloroform and the chloroform extract, after drying over anhydrous sodium sulfate, concentrated under diminished pressure to a syrup. The product of five such methylations was combined, dissolved in ether, dried with anhydrous sodium sulfate, and subjected to a fractional distillation.

The largest fraction amounting to 90 gm. boiled at 88–90° at a pressure of 0.03 mm.

0.1025 gm. of substance gave on combustion 0.2042 gm. of CO<sub>2</sub> and 0.0780 gm. of H<sub>2</sub>O.

C <sub>12</sub> H <sub>22</sub> O <sub>6</sub> .	Calculated.	C 54.96, H 8.17.
	Found.	C 54.33, H 8.5.

The optical rotation was:

$$[\alpha]_D^{20} = \frac{-1.15^\circ \times 100}{1 \times 4.034} = -28.5^\circ$$

---

<sup>4</sup> Irvine, J. C., and Scott, J. P., *J. Chem. Soc.*, 1913, ciii, 573.

*3-5-6-Trimethyl Glucose.*

Hydrolysis of the trimethyl acetone glucose was carried out in 75 per cent alcoholic solution containing 0.5 per cent hydrochloric acid and heating for 1 hour at 100°. The solution was shaken with silver carbonate, extracted with ether, and the ethereal solution dried and concentrated to a syrup. This was distilled and a fraction obtained boiling at 147° and  $P = 0.05$  mm.

0.1150 gm. of substance gave on combustion 0.2080 gm. of  $\text{CO}_2$  and 0.0872 gm. of  $\text{H}_2\text{O}$ .

$\text{C}_9\text{H}_{19}\text{O}_6$ .	Calculated.	C 48.75, H 8.11.
	Found.	C 49.32, H 8.43.

The optical rotation in alcohol was:

Initial.	Final.
$[\alpha]_D^{20} = \frac{-0.75^\circ \times 100}{1 \times 6.874} = -10.95^\circ$	$[\alpha]_D^{20} = \frac{-1^\circ \times 100}{1 \times 6.874} = -14.6^\circ$

It was attempted to convert this product into its methyl glucoside by all the known methods and by several modifications as regards the concentration of catalyst and time of reaction. In every experiment the unchanged material was recovered.

*3-5-6-Trimethyl-Methyl Glucoside.*

The conversion of trimethyl monoacetone glucose to the glucoside was obtained finally by the following process:

50 gm. of trimethyl acetone glucose were dissolved in 100 cc. of dry methyl alcohol containing 0.1 per cent hydrochloric acid and heated in sealed tubes for 24 hours at 100°C. The hydrochloric acid was removed by shaking with moist silver carbonate and the solution concentrated under diminished pressure to a syrup. This was taken up in ether and dried with anhydrous sodium sulfate. The ethereal solution was concentrated and the syrup fractionated. The larger part distils at 135°,  $P = 0.035$  mm. The yield was 20 gm.

0.1510 gm. of substance gave on combustion 0.2758 gm. of  $\text{CO}_2$  and 0.1174 gm. of  $\text{H}_2\text{O}$ .

$\text{C}_{10}\text{H}_{21}\text{O}_6$ .	Calculated.	C 50.85, H 8.47.
	Found.	C 49.8, H 8.7.

Repeated attempts to obtain this glucoside uncontaminated with traces of free sugar were unsuccessful. The product always produced a slight reduction of Fehling's solution.

*3-5-6-Trimethyl-2-Phosphoric Acid Methyl Glucoside.*

10 gm. of 3-5-6-trimethyl-methyl glucoside were dissolved in dry pyridine, cooled to  $-20^{\circ}$ , and a solution of 5.1 gm. of phosphorus oxychloride in 20 cc. of pyridine, also cooled to  $-20^{\circ}$ , was slowly added. There was a slight rise in temperature and after standing at  $-10^{\circ}$  for some time, crystals of pyridine hydrochloride separated. The barium salt, prepared by the method previously outlined, was found to be soluble in alcohol, ether, and acetone. The alcoholic solution was allowed to evaporate. The barium salt which was obtained as a white powder did not reduce Fehling's solution until after hydrolysis with acid.

For analysis the substance was dried under diminished pressure at the temperature of water vapor.

0.1070 gm. of substance gave on combustion 0.1258 gm. of  $\text{CO}_2$  and 0.0570 gm. of  $\text{H}_2\text{O}$ .

0.2918 gm. of substance gave 0.0717 gm. of  $\text{Mg}_2\text{P}_2\text{O}_7$ .

0.0973 " " " " 0.0274 " "  $\text{BaSO}_4$ .

$\text{C}_{10}\text{H}_{19}\text{O}_6\text{H}_2\text{PO}_3$ . Calculated. C 37.97, H 6.63, P 9.80.

Found (calculated Ba-free). C 38.6, H 7.15, P 8.25.

The barium salt was in the main an acid barium salt, since analysis showed 16.5 per cent of barium; theory requires for the acid salt 17.85 per cent, and for the neutral salt 30.5 per cent barium.

The optical rotation in water was:

$$[\alpha]_D^{20} = \frac{0.71^{\circ} \times 100}{1 \times 2.692} = +26.38^{\circ}$$

*Rates of Hydrolysis.*

*1-2-Monoacetone-3- or 5-Phosphoric Acid Glucose.*

1.658 gm. of the barium salt of this substance were dissolved in a small volume of warm water and made up to 50 cc. Of this solution 3 cc., equivalent to 0.030 gm. of P, were put into glass tubes together with 2.832 cc. of 0.1 N  $\text{H}_2\text{SO}_4$  and 0.168 cc. of water and sealed. The tubes were heated in an oil bath at 100 cc.

for the intervals indicated in the following tables. The method of analysis was as described in the paper of Levene and Yamagawa.<sup>5</sup>

*3-5-6-Trimethyl-2-Phosphoric Acid Methyl Glucoside.*

3.651 gm. of the barium salt of this substance were dissolved in a little warm water and made up to 25 cc. Of this solution 3 cc., equivalent to 0.030 gm. of P, were put into glass tubes together with 1.227 cc. of 0.1 N H<sub>2</sub>SO<sub>4</sub> and 0.773 cc. of water and sealed. The tubes were heated at 100° for the intervals indicated in the following tables.

*1-2-Monoacetonc-3- or 5-Phosphoric Acid Glucose.*

Time.	Mg <sub>2</sub> P <sub>2</sub> O <sub>7</sub>	Average.	P in Mg <sub>2</sub> P <sub>2</sub> O <sub>7</sub> .	P in free acid.	P of total P.
<i>hrs.</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>per cent</i>	<i>per cent</i>
1	0.0014	0.0013	0.0009	0.27	3.02
	0.0012				
2	0.0030	0.0032	0.0022	0.67	7.42
	0.0034				
4	0.0062	0.0063	0.0044	1.33	14.61
	0.0064				
6	0.0082	0.0083	0.0059	1.75	19.25
	0.0083				
8	0.0099	0.0100	0.0070	2.11	23.19
	0.0100				
16	0.0160	0.0161	0.0115	3.47	38.21
	0.0162				
24	0.0199	0.0199	0.0139	4.20	46.16
	0.0198				

<sup>5</sup> Levene, P. A., and Yamagawa, M., *J. Biol. Chem.*, 1920, xliii, 323.

*3-5-6-Trimethyl-2-Phosphoric Acid Methyl Glucoside.*

Time.	Mg <sub>2</sub> P <sub>2</sub> O <sub>7</sub>	Average.	P in Mg <sub>2</sub> P <sub>2</sub> O <sub>7</sub>	P in free acid.	P of total P.
<i>hrs.</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>per cent</i>	<i>per cent</i>
1	0.0178	0.0179	0.0125	3.37	41.53
	0.0179				
2	0.0210	0.0213	0.0151	4.09	50.31
	0.0214				
4	0.0210	0.0210	0.0146	3.96	48.71
	0.0209				
6	0.0232	0.0233	0.0162	4.39	54.05
	0.0234				
8	0.0207	0.0208	0.0145	3.92	48.25
	0.0208				
16	0.0212	0.0213	0.0148	4.01	49.41
	0.0214				

## Sample 2.

1	0.0303	0.0302	0.0084	4.46	70.04
	0.0301				
2	0.0303	0.0305	0.0085	4.50	70.74
	0.0307				
4	0.0320	0.0324	0.0090	4.89	76.90
	0.0328				
6	0.0348	0.0346	0.0096	5.11	80.26
	0.0344				

*1-2-Monoacetone-3- or 5-Phosphoric Acid Glucose.*

<i>T</i>	Mg <sub>2</sub> P <sub>2</sub> O <sub>7</sub> ( <i>x</i> )	<i>a</i> - <i>x</i>	$\frac{1}{t} \log \frac{a}{a-x}$
<i>min.</i>	<i>gm.</i>		
60	0.0033	0.1044	0.00023
120	0.0080	0.0997	0.00028
240	0.0157	0.0920	0.00028
360	0.0208	0.0869	0.00026
480	0.0250	0.0827	0.00024
960	0.0402	0.0675	0.00021
1,440	0.0500	0.0577	0.00020
Average .....			0.00024

$$a = 0.1077$$

*3-5-6-Trimethyl-2-Phosphoric Acid Methyl Glucoside.*

<i>T</i>	Mg <sub>2</sub> P <sub>2</sub> O <sub>7</sub> ( <i>x</i> )	<i>a</i> - <i>x</i>	$\frac{1}{t} \log \frac{a}{a-x}$
<i>min.</i>	<i>gm.</i>		
60	0.0448	0.0629	0.0038
120	0.0530	0.0547	0.0024
240	0.0525	0.0552	0.0012
360	0.0583	0.0494	0.0009
480	0.0520	0.0557	0.0006
960	0.0533	0.0533	0.0003
1,440	0.0692	0.0385	0.0003

$$a = 0.1077$$

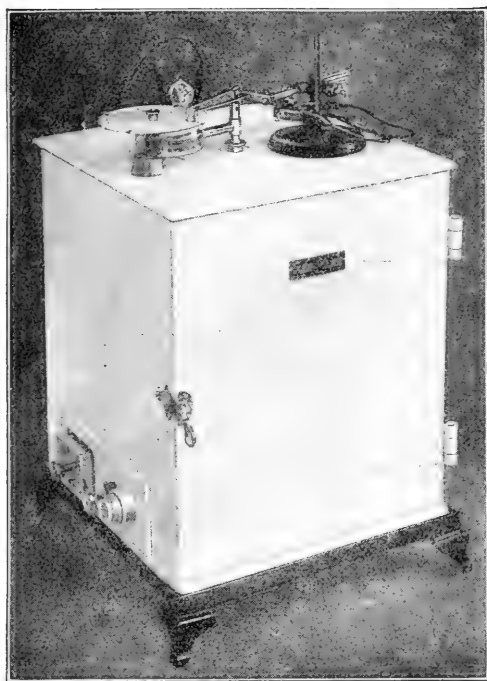
*3-5-6-Trimethyl-2-Phosphoric Acid Methyl Glucoside.*

## Sample 2.

<i>T</i>	Mg <sub>2</sub> P <sub>2</sub> O <sub>7</sub> ( <i>x</i> )	<i>a</i> - <i>x</i>	$\frac{1}{t} \log \frac{a}{a-x}$
<i>min.</i>	<i>gm.</i>		
60	0.0755	0.0322	0.0087
120	0.0763	0.0314	0.0044
240	0.0829	0.0248	0.0026
360	0.0865	0.0212	0.0017

$$a = 0.1077$$

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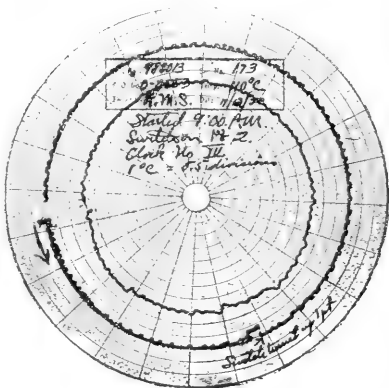
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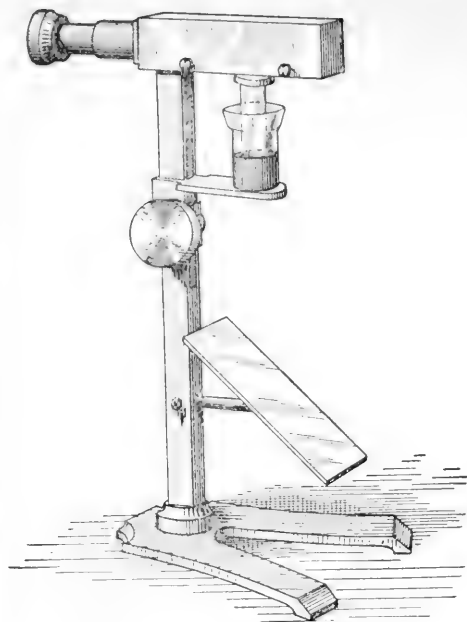
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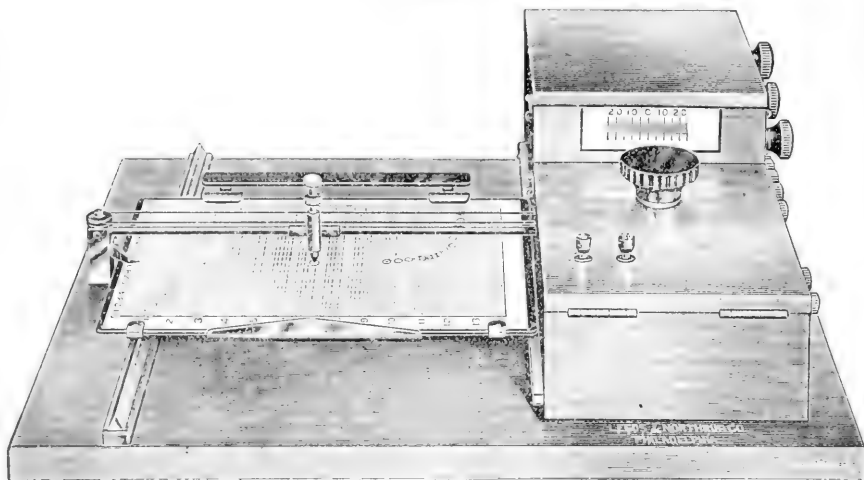
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## THE QUANTITATIVE DETERMINATION OF AMINO-ACIDS OF FEEDS.

By T. S. HAMILTON, W. B. NEVENS, AND H. S. GRINDLEY.

(From the Department of Animal Husbandry, University of Illinois, Urbana.)

(Received for publication, July 12, 1921.)

### INTRODUCTION.

The need and the importance of knowledge concerning the amino-acid content of foods and feeds require no emphasis. Grindley, Joseph, and Slater (1) in 1915 were the first to publish data on the quantitative determination of amino-acids in feeds. About 1 month later Nollau (2) published results on the amino-acid contents of certain commercial feeds. Later in the same year Grindley and Slater (3), in a second paper, published additional results on the same problem. Both Grindley and associates and Nollau used in their work the Van Slyke method, but the results from the two laboratories do not agree well. As is stated in more detail later, the lack of concordant results, in general, may be explained by differences in the procedures used.

Criticism has been made, however, of the application of a method, designed entirely for the purpose of analyzing pure isolated proteins, to the analysis of heterogeneous mixtures such as feeds. Among several difficulties mentioned, the effects of the non-protein nitrogenous constituents and of the carbohydrates on the results of the Van Slyke analysis were unknown. In order to determine the effect of the non-protein nitrogenous material, Grindley and Eckstein (4) made a study of the non-protein constituents extracted from various feeds with cold water. Hart and Bentley (5) made a similar study but used hot water instead of cold water as their extracting fluid. From the fact that most of the non-protein nitrogen of the feeds examined was in the form of ammonia or  $\alpha$ -amino-acid nitrogen, free or combined, Grindley and Eckstein in part conclude:

" . . . it seems quite evident that only a small part, if any, of the nonprotein nitrogenous constituents of foods and feeding stuffs can in anyway interfere with the application of the Van Slyke method for the determination of the chemical groups characteristic of the different amino acids of protein to the estimation of the free and combined amino acids and amides of feeding stuffs."

And that no claim to perfection is made for the results published by Grindley and his associates is shown by the statement of Grindley (6): "Further it is also quite evident that the results so far obtained in this work are only approximately accurate and at present are to be considered of comparative value only."

The chief source of error in the method of analysis used by Grindley and associates was thought to be caused by the presence of the carbohydrates in the feeds during the hydrolysis and subsequent analysis. Gortner and his associates (7, 8) have made an extensive study of the formation of humin in the presence of carbohydrates during acid hydrolysis. These authors have shown that the formation of humin depends to a large extent on the presence of carbohydrates and that the quantity of humin formed on hydrolysis of pure proteins is greatly increased by the addition of carbohydrate material. Attempting to reduce the quantity of humin formed during hydrolysis, Eckstein and Grindley (9) made two decided improvements on the older method. The first was the removal of some of the non-protein nitrogenous constituents by extractions with ether and cold absolute alcohol. The second was "the conversion, as far as possible, of the insoluble carbohydrates into soluble carbohydrates by boiling the feeding-stuffs with 0.1 per cent hydrochloric acid." In this manner it was possible to separate a large part of the carbohydrates from the main portion of the proteins before the latter are hydrolyzed. The quantity of humin nitrogen obtained by this method compared very favorably with that formed in the analysis of some of the pure proteins.

While this method of Eckstein and Grindley was a decided improvement over the first method used by Grindley and associates, it was far from perfect. The perfection of a method for the quantitative determination of the amino-acid content of feeds has been the aim of an extensive investigation conducted in this laboratory.

*Method.*

The method outlined below is the culmination of 146 experiments designed to separate the proteins of feeds quantitatively, either as such or as hydrolyzed proteins, from the other constituents of the sample which would interfere with the determination of amino-acids by the Van Slyke method (10,11). The completeness with which this has been accomplished is shown by an examination of Tables I and II.

The analysis is divided into two distinct parts: First, the treatment of a sample of feed so that all the proteins are obtained in solutions sufficiently free from interfering substances so that the Van Slyke method for the estimation of certain amino-acids may be applied; and second, the quantitative estimation of these amino-acids.

The first part of the procedure consists of a series of extractions with various solvents, and, in those extracts in which it is necessary, the separation of the proteins, by various methods, from the interfering substances. The residue remaining after the last extraction consists chiefly of crude fiber and contains very little nitrogen. The essential features of this part of the procedure are as follows:

1. The non-protein nitrogenous constituents are extracted from a weighed quantity of the finely ground feed, equivalent to approximately 6 gm. of protein, by extracting with anhydrous ether, cold absolute ethyl alcohol, and cold 1.0 per cent trichloroacetic acid, in the order named. These extractions as well as all other extractions in the cold are carried out in the following manner: The sample is placed in a 500 cc. centrifuge bottle, 100 to 200 cc. of the extracting liquid are added, and the bottle is placed on a shaker arrangement which rolls the bottle back and forth continuously. Usually but two extractions are made each 24 hours; one extraction for a 7 to 8 hour period is made during the day and a second extraction for a 14 to 15 hour period is made during the night. As a rule six or seven extractions with each solvent are necessary to insure complete extractions in the cold. After each extraction period the solution is centrifuged and the supernatant liquid decanted.

The small amount of protein extracted by the cold trichloroacetic acid is recovered by precipitation with colloidal ferric hydrate.

2. The main portion of the proteins is next extracted with cold dilute sodium hydroxide solution on the shaker. A 0.2 per cent solution of sodium hydroxide is used as the extracting liquid during the day and a 0.1 per cent solution is used during the longer night period. After the sixth extraction with alkali the residue is washed a few times with 100 cc. portions of ammonia-free water to remove the alkali.

3. The starch is next removed from the residue by extracting with hot 2.0 per cent trichloroacetic acid. The residue from the dilute alkali extraction is transferred to a round bottom digestion flask with 500 cc. of 2.0 per cent trichloroacetic acid. The flask is placed on a boiling steam bath and with frequent shakings allowed to digest until it is apparent (from the disappearance of the milky color) that much of the starch has been dissolved. The solution is then filtered, washed with hot water, and the residue treated again with 250 cc. of the 2.0 per cent trichloroacetic acid. A third digestion with 250 cc. of the acid generally completes the extraction of starch. A small amount of protein is extracted by the trichloroacetic acid and this is separated from the starch by concentrating the united trichloroacetic acid filtrates under diminished pressure to about one-third their original volume and precipitating the starch with two volumes of alcohol. Only a negligible amount of nitrogen is found in the starch thus precipitated, while the filtrate is practically starch-free.

4. The residue from the above treatment is next boiled with 250 cc. of 20 per cent hydrochloric acid for 3 minutes, cooled, filtered, and washed with ammonia-free water (keeping the washings separate from the 20 per cent HCl filtrate). The residue is again treated with 250 cc. of 20 per cent HCl in exactly the same manner as before.

5. The residue from the 20 per cent HCl treatment is transferred to a centrifuge bottle and extracted for three 24 hour periods on the shaker with 50 cc. portions of 5 per cent sodium hydroxide solution.

The entire protein content of the sample is found, from the above procedure, in the following fractions: (a) the colloidal ferric hydrate precipitate from the cold 1.0 per cent trichloroacetic acid extract; (b) the dilute alkali extract; (c) the filtrate from the alcoholic precipitation of the starch; (d) the 20 per cent hydro-

chloric acid extract; and (e) the strong alkali extract. The colloidal ferric hydrate precipitate is transferred to a digestion flask with 20 per cent hydrochloric acid; the dilute alkali extract is neutralized, concentrated under diminished pressure to a small volume, and then transferred to a digestion flask with an equal volume of concentrated hydrochloric acid; the alcoholic filtrate is concentrated under diminished pressure to remove the alcohol, then transferred to a digestion flask with an equal volume of concentrated hydrochloric acid; the filtrate from the extraction with boiling 20 per cent hydrochloric acid and the washings are transferred to a digestion flask with a volume of concentrated hydrochloric acid equal to the volume of the washings; the strong alkali extract is neutralized, concentrated under diminished pressure to a small volume, and then transferred to a digestion flask with an equal volume of concentrated hydrochloric acid. The proteins in these fractions are completely hydrolyzed by boiling for 15 to 20 hours under reflux condensers.

The completely hydrolyzed protein solutions are combined and analyzed according to the Van Slyke method. Some slight modifications, most of which were made necessary by the larger amount of protein material present, have been made. A few other minor changes in the technique have been found advisable in the application of the original method to this type of solution.

Four samples are usually run at the same time. The following chemical methods are used: Van Slyke's method for amino-acid nitrogen and ammonia (amide nitrogen), Plimmer's method (12) for arginine, Denis' modification of Benedict's method for organic sulfur, and the Gunning-Arnold-Dyer modification of the Kjeldahl method for total nitrogen. Duplicate or triplicate determinations were always made whenever possible. The histidine and lysine nitrogen are calculated according to Van Slyke's original method (10)<sup>1</sup>.

<sup>1</sup> The error in Van Slyke's formula for the calculation of histidine nitrogen has been corrected. Instead of

$$\text{Histidine N} = \frac{3}{2} (D - \frac{3}{4} \text{ Arg.}) = 1.007D - 1.125 \text{ Arg.}$$

we have always used,

$$\text{Histidine N} = \frac{3}{2} (D - \frac{3}{4} \text{ Arg.}) = 1.5D - 1.125 \text{ Arg.}$$

## DISCUSSION.

Tables I and II give the results of 6 complete analyses of oats, 8 complete analyses of cottonseed meal, 4 of alfalfa, and 6 of corn. In Table I the results are expressed as percentages of total nitrogen in the feed and in Table II as percentages of the feed. The oats contained 1.680 per cent nitrogen, the cottonseed meal 6.796 per cent, the alfalfa 2.628 per cent, and the corn 1.4074 per cent. The weights of feeds taken for each of the various analyses were 60 gm. in the case of oats and corn, 30 gm. in the case of alfalfa, and 15 gm. in the case of cottonseed meal. The oats and corn were ground so as to pass through an 80 mesh sieve, the alfalfa through a 60 mesh sieve, and the cottonseed meal through a 40 mesh sieve.

Although a comparison of the four feeds, from a standpoint of nitrogen distribution, is not intended at this time, attention may be called to a few of the more significant figures, mainly in support of the method. A brief examination of Tables I and II will show that all solutions, residues, precipitates, and other fractions, obtained in the preparation of the hydrolyzed protein solution and in the subsequent Van Slyke analysis of that solution, were analyzed for their nitrogen content. In other words, no fraction, which might in any way contain any portion of the original sample of feed taken for analysis, was discarded without its total nitrogen content having first been determined. This fact should be kept in mind when a comparison of these results is made with those of other workers.

For convenience, our results on the distribution of nitrogen in the feed, are divided into non-protein nitrogen, nitrogen distribution as shown by the Van Slyke analysis, and the nitrogen lost in the method of analysis.

Criticism has been made of the application of the Van Slyke method to the analysis of feeds on the basis of the possible interference of some of the non-protein nitrogenous constituents. In the present method the ether and the alcohol extractions of Eckstein and Grindley (9) are followed by a cold 1.0 per cent trichloroacetic acid extraction. Trichloroacetic acid, being a comparatively strong acid, and also, in dilute solutions, a good protein precipitant, was found to extract the remaining non-protein



nitrogenous substances quite readily, while only a very small amount of protein material was extracted. This small quantity of protein is separated from the non-protein constituents by precipitation with colloidal ferric hydrate<sup>2</sup>.

Although the exact rôle, played in protein metabolism by all of the non-protein nitrogenous constituents of a feed, is at present unknown, it is of interest to note that these four feeds vary markedly in their non-protein nitrogen content. Alfalfa has the highest percentage of non-protein nitrogen with an average of 19.09 per cent, while cottonseed meal has the lowest with an average of 6.20 per cent. Oats and corn have intermediate values of 12.93 and 9.83 per cent, respectively. Judging from these values, which agree very well with those of Grindley and Eckstein (4), roughages have a higher non-protein nitrogen content and concentrates a lower content than the cereals.

Under the heading "nitrogen lost in method of analysis" are found the results of the total nitrogen determinations on the various fractions, the amino-acid content of which escapes analysis by our method of preparing the hydrolyzed protein solution and by the Van Slyke analysis of this hydrolyzed protein solution. The total nitrogen lost is shown in Column U. The nitrogen lost in the preparation of the hydrolyzed protein solution is shown in Column O, representing the nitrogen left in the residue after treatment with the last extracting fluid, 5 per cent NaOH, and in Column P, giving the nitrogen in the alcohol precipitate (starch) of the hot 2 per cent trichloroacetic acid extract. The extraction of starch was not made in the cases of cottonseed meal and alfalfa because of the small amounts present.

The nitrogen lost in the analysis of the hydrolyzed protein solution by the Van Slyke method, consists of (a) the "unadsorbed humin" of Van Slyke (11), filtered from the amyl alcohol-ether aqueous solution during the decomposition of the bases; (b) the nitrogen extracted by the amyl alcohol-ether mixture; (c) the nitrogen in the residue filtered from the solution of the bases; and (d) the nitrogen in the residue filtered from the solution of the filtrate from the bases. As percentages of the total nitrogen of the feed, the total nitrogen lost, as shown in Column U, Table I, is 1.90 per cent in the case of oats, 3.29 per cent in cottonseed meal, 3.85 per cent in corn, and 4.73 per cent in alfalfa.

<sup>2</sup> Dialyzed iron Merck, containing 5 per cent  $\text{Fe}_2\text{O}_3$ .

TABLE I.—*Distribution of the Nitrogen of Oats, Corn, Cottonseed*

	Non-protein nitrogen.										Results of
	Soluble in ether.	Soluble in alcohol.	In filtrate from colloidal iron.	Total non-protein N.	Insoluble humin N.	Soluble humin N.	Acid amide N.	Arginine N.*	Cystine N.*	Histidine N.*	
Oats (containing 10% water)											
	A	B	C	D	E	F	G	H	I	J	
	0.655	1.172	11.522	13.349	2.873	2.783	11.016	11.427	0.961	6.50	
	0.597	1.163	11.126	12.886	2.968	3.231	11.083	11.652	1.008	5.65	
	0.553	1.041	11.766	13.360	3.481	1.498	11.780	11.888	0.976	5.54	
	0.555	1.375	11.140	13.070	2.893	1.748	11.566	11.474	0.951	5.94	
	0.513	1.307	10.360	12.180	2.611	2.914	11.350	11.892	0.894	5.84	
	0.544	1.294	10.860	12.698	3.255	2.926	11.738	11.554	0.876	5.28	
Average.....	0.569	1.225	11.129	12.926	3.013	2.516	11.422	11.647	0.944	5.79	
Corn† (containing 10% water)											
	0.050	0.997	9.311	10.358	1.571	1.375	11.729	8.620	1.099	5.24	
	0.792	2.341	8.093	11.226	1.796	2.602	12.265	8.868	1.165	5.46	
	0.658	2.369	8.395	11.422	1.499	2.284	12.225	8.867	1.186	4.90	
	0.240	0.239	6.836	7.315	0.702	2.685	11.241	8.782	0.925	4.74	
	0.028	0.305	7.816	8.149	0.758	2.596	12.218	8.762	0.985	3.92	
	0.189	1.958	8.356	10.503	1.084	2.278	9.833‡	8.451	1.071	4.70	
Average.....	0.326	1.368	8.135	9.829	1.235	2.303	11.936	8.725	1.072	4.83	
Cottonseed meal											
	0.021	0.570	4.943	5.534	2.609	3.462	9.455	18.672	0.961	5.48	
	0.089	0.618	4.870	5.577	2.609	5.117	9.689	19.050	0.902	6.33	
	0.202	0.652	5.053	5.907	2.492	5.459	9.929	18.467	1.068	7.54	
	0.109	0.614	5.531	6.254	2.623	4.477	8.892	18.398	1.123	7.24	
	0.081	0.506	5.245	5.832	2.981	2.415	9.249	17.520	1.051	8.58	
	0.129	0.489	5.722	6.340	2.930	2.650	9.318	19.443	0.948	6.36	
	0.081	0.420	6.097	6.598	2.763	2.334	9.002	17.987	0.707	9.35	
	0.046	0.506	7.012	7.564	2.772	2.746	9.764	20.102	0.781	6.46	
Average.....	0.095	0.547	5.559	6.201	2.722	3.582	9.412	18.705	0.943	7.17	

\*Corrected for solubility of the bases.

† The authors are indebted chiefly to Nao Uyei, assistant chemist, for the following.

‡ Not included in the average.

§ Determination lost; average result substituted to make up total.

Real, and Alfalfa (Expressed as Percentage of Total Nitrogen).

Van Slyke analysis.			Nitrogen lost in method of analysis.							Total.	
Lysine N*.	Amino-acid N in filtrate from bases.*	Non-amino-acid N in filtrate from bases.*	Total non-protein + results of Van Slyke analysis.	N in residue after treatment with strong NaOH.	In alcohol precipitate of hot 2 per cent CCl <sub>4</sub> . CO <sub>2</sub> H extracts.	Unadsorbed humin (filtered from solution during decomposition of bases).	In amyl alcohol-ether extract.	In residue filtered from solution of bases.	In residue filtered from solution of filtrate from bases.	Total N lost.	Total N accounted for.

680 per cent N).

K	L	M	N	O	P	Q	R	S	T	U	V
182	41.992	4.108	97.195	0.120	0.109	0.558	0.525	0.363	0.024	1.699	98.894
821	41.981	2.964	96.245	0.055	0.108	0.835	0.771	0.297	0.033	2.099	98.344
445	42.174	3.809	97.954	0.136	0.148	0.566	0.759	0.083	0.015	1.707	99.661
386	41.928	4.988	96.946	0.165	0.123	0.851	0.929	0.141	0.025	2.234	99.180
426	42.066	3.588	96.761	0.148	0.153	0.340	0.755	0.191	0.020	1.607	98.368
787	42.682	3.704	97.503	0.168	0.123	0.833	0.738	0.179	0.033	2.074	99.577
841	42.137	3.860	97.100	0.132	0.127	0.664	0.746	0.209	0.025	1.903	99.004

4074 per cent N).

424	46.090	5.600	94.111	0.505‡	0.513	0.930	0.521	0.189	0.065	2.723	96.834
418	46.790	0.884‡	93.474	0.176	0.163	0.930	0.527	0.206	1.468‡	3.470	96.944
473	45.059	8.251	98.172	0.147	0.335	1.999	0.515	0.230	0.008	3.234	101.406
847	47.750	8.763	94.758	0.107	0.265	3.686	0.372	0.149	0.133	4.712	99.470
221	47.962	6.870	94.449	0.113	0.073	2.791	0.531	0.164	0.045	3.717	98.166
814	46.574	6.599	92.909	0.137	0.305	5.850	0.418	0.209	0.075	6.994	99.903
200	46.704	7.216	96.052	0.136	0.276	2.698	0.481	0.191	0.065	3.847	99.899

contains 6.796 per cent N).

240	39.981	3.271	93.671	0.220	Extraction not made.	2.274	0.980	0.206	0.036	3.716	97.387
060	40.539	3.432	96.305	0.260		1.562	0.906	0.215	0.039	2.982	99.287
570	38.852	1.901	95.188	0.302		1.658	0.788	0.220	0.106	3.074	98.262
470	39.828	0.161‡	93.466	0.233		3.044	1.135	0.245	0.130	4.787	98.253
462	41.958	2.681	96.733	0.430§		1.192	0.691	0.428	0.150	2.891	99.624
998	41.950	2.290	97.233	0.685		1.019	0.780	0.380	0.096	2.960	100.193
600	39.204	3.087	94.633	0.719		0.772	1.026	0.096	0.065	2.678	97.311
274	43.481	3.454	101.402	0.589		1.364	1.068	0.133	0.068	3.222	105.624
209	40.724	2.874	96.543	0.430		1.611	0.922	0.240	0.086	3.289	99.832

Analyses of corn.

TABLE

Non-protein nitrogen.					Result of t					
	Soluble in ether.	Soluble in alcohol.	In filtrate from colloidal iron.	Total non protein N.	Insoluble humin N.	Soluble humin N.	Acid amide N.	Arginine N.*	Cystine N.*	Histidine N.*
Alfalfa (contain										
	A	B	C	D	E	F	G	H	I	J
	0.577	1.940	16.466	18.983	3.682	4.483	6.943	7.949	0.924	4.35
	0.577	1.600	16.301	18.478	3.690	3.512	7.104	8.064	1.062	3.65
	0.524	1.988	17.289	19.801	3.597	5.132	8.204	7.523	0.986	3.78
	0.522	1.864	16.712	19.098	3.791	4.796	7.204	8.446	0.991	3.93
Average.....	0.550	1.848	16.692	19.090	3.690	4.481	7.364	7.996	0.991	3.93

oncluded.

Van Slyke analysis.			Nitrogen lost in method of analysis.							Total.	
Lysine N.*	Amino-acid N in filtrate from bases.*	Non-amino-acid N in filtrate from bases.*	Total non-protein + results of Van Slyke analysis.	N in residue after treatment with strong NaOH.	In alcohol precipitate of hot 2 per cent $\text{CCl}_3$ . $\text{CO}_2\text{H}$ extracts.	Unadsorbed humin (filtered from solution during decomposition of bases).	In amyl alcohol-ether extract.	In residue filtered from solution of bases.	In residue filtered from solution of filtrate from bases.	Total N lost.	Total N accounted for.

328 per cent N).

K	L	M	N	O	P	Q	R	S	T	U	V
334	38.349	3.863	93.866	2.663	Extraction not made.	0.999	0.609	Not detected.	0.144	4.415	98.281
959	37.681	3.059	91.264	2.335		1.110	0.816		0.639	4.900	96.164
008	37.312	1.196	91.541	2.930		1.274	0.301		0.604	5.109	96.650
434§	38.786	1.927	93.404	2.146		1.261	0.717		0.377	4.501	97.905
434	38.032	2.511	92.520	2.519		1.161	0.611		0.441	4.732	97.252

TABLE II.—*Distribution of the Nitrogen of Oats, Corn, Cotton*

Non-protein nitrogen.				Results of Van Slyke							
Soluble in ether.	Soluble in alcohol.	In filtrate from colloidal iron.	Total non-protein N.	Insoluble humin N.	Soluble humin N.	Acid amide N.	Arginine N.*	Cystine N.*	Histidine N.*	Lysine N.*	
Oats (containing 10.0% N.)											
A	B	C	D	E	F	G	H	I	J	K	
0.0110	0.0197	0.1936	0.2242	0.0482	0.0467	0.1850	0.1919	0.0161	0.1092	0.0366	
0.0100	0.0195	0.1869	0.2164	0.0498	0.0542	0.1862	0.1957	0.0169	0.0949	0.0474	
0.0093	0.0175	0.1977	0.2244	0.0584	0.0251	0.1979	0.1997	0.0164	0.0931	0.0578	
0.0093	0.0231	0.1872	0.2195	0.0486	0.0293	0.1943	0.1927	0.0159	0.0998	0.0400	
0.0086	0.0220	0.1740	0.2046	0.0438	0.0489	0.1906	0.1997	0.0150	0.0981	0.0575	
0.0091	0.0217	0.1824	0.2133	0.0546	0.0491	0.1972	0.1941	0.0147	0.0887	0.0468	
Average	0.0096	0.0206	0.1870	0.2170	0.0506	0.0422	0.1918	0.1956	0.0158	0.0973	0.0477
Corn† (containing 10.0% N.)											
0.0007	0.0140	0.1310	0.1458	0.0221	0.0194	0.1651	0.1213	0.0155	0.0738	0.0341	
0.0111	0.0330	0.1139	0.1580	0.0253	0.0366	0.1726	0.1248	0.0164	0.0768	0.0340	
0.0093	0.0333	0.1182	0.1608	0.0211	0.0321	0.1721	0.1248	0.0167	0.0690	0.0348	
0.0034	0.0034	0.0962	0.1030	0.0099	0.0378	0.1582	0.1236	0.0130	0.0668	0.0260	
0.0004	0.0043	0.1100	0.1147	0.0107	0.0365	0.1720	0.1233	0.0139	0.0553	0.0313	
0.0027	0.0276	0.1176	0.1478	0.0153	0.0321	0.1384‡	0.1189	0.0151	0.0662	0.0255	
Average	0.0046	0.0193	0.1145	0.1383	0.0174	0.0324	0.1680	0.1228	0.0151	0.0680	0.0310
Cottonseed meal											
0.0014	0.0387	0.3360	0.3761	0.1773	0.2352	0.6426	1.2689	0.0653	0.3728	0.2882	
0.0061	0.0420	0.3310	0.3790	0.1773	0.3478	0.6585	1.2946	0.0613	0.4302	0.2080	
0.0137	0.0443	0.3434	0.4014	0.1694	0.3710	0.6748	1.2550	0.0726	0.5126	0.2426	
0.0075	0.0417	0.3459	0.4250	0.1783	0.3043	0.6043	1.2503	0.0763	0.4920	0.3038	
0.0055	0.0345	0.3565	0.3963	0.2026	0.1641	0.6286	1.1906	0.0714	0.5834	0.3032	
0.0088	0.0332	0.3889	0.4309	0.1991	0.1801	0.6333	1.3213	0.0644	0.4326	0.3399	
0.0055	0.0285	0.4144	0.4484	0.1878	0.1586	0.6118	1.2224	0.0480	0.6356	0.2447	
0.0032	0.0344	0.4765	0.5140	0.1884	0.1866	0.6635	1.3661	0.0631	0.4393	0.2905	
Average	0.0065	0.0372	0.3778	0.4214	0.1850	0.2434	0.6396	1.2712	0.0641	0.4873	0.2860

\* Corrected for solubility of the bases.

† The authors are indebted chiefly to Nao Uyei, assistant chemist, for the follow-

‡ Not included in the average.

§ Determination lost; average result substituted to make up total.

seed Meal, and Alfalfa (Expressed as Percentage of Feed).

analysis.		Total non-protein + results of Van Slyke analysis.	Nitrogen lost in method of analysis.							Total.
Amino-acid N in filtrate from bases.*	Non-amino acid N in filtrate from bases.*		N in residue after treatment with strong NaOH.	In alcohol precipitate of hot 2 per cent CCl <sub>3</sub> . CO <sub>2</sub> H extracts.	Unadsorbed humin (filtered from solution during decomposition of bases).	In amyl alcohol-ether extract.	In residue filtered from solution of bases.	In residue filtered from solution of filtrate from bases.	Total N lost.	Total N accounted for.
.680 per cent N).										
L	M	N	O	P	Q	R	S	T	U	V
0.7054	0.0690	1.6328	0.0020	0.0018	0.0094	0.0088	0.0061	0.0004	0.0285	1.6614
0.7052	0.0498	1.6169	0.0009	0.0018	0.0140	0.0130	0.0050	0.0006	0.0352	1.6521
0.7085	0.0640	1.6456	0.0023	0.0025	0.0095	0.0128	0.0014	0.0003	0.0286	1.6743
0.7044	0.0838	1.6287	0.0028	0.0021	0.0143	0.0156	0.0024	0.0004	0.0375	1.6662
0.7067	0.0602	1.6255	0.0025	0.0026	0.0057	0.0127	0.0032	0.0003	0.0270	1.6525
0.7071	0.0622	1.6380	0.0028	0.0021	0.0140	0.0124	0.0030	0.0006	0.0348	1.6729
0.7079	0.0648	1.6312	0.0022	0.0021	0.0112	0.0125	0.0035	0.0004	0.0319	1.6632
.4074 per cent N).										
0.6487	0.0788	1.3245	0.0071‡	0.0072	0.0131	0.0073	0.0027	0.0009	0.0383	1.3628
0.6585	0.0124	1.3156	0.0025	0.0023	0.0131	0.0074	0.0029	0.0207	0.0488	1.3644
0.6342	0.1161	1.3817	0.0021	0.0047	0.0281	0.0072	0.0032	0.0001	0.0455	1.4271
0.6720	0.1233	1.3336	0.0015	0.0037	0.0519	0.0052	0.0021	0.0019	0.0663	1.3999
0.6750	0.0967	1.3293	0.0016	0.0010	0.0393	0.0075	0.0023	0.0006	0.0523	1.3816
0.6555	0.0929	1.3076	0.0019	0.0043	0.0823	0.0059	0.0029	0.0011	0.0984	1.4060
0.6573	0.1016	1.3518	0.0019	0.0039	0.0380	0.0068	0.0027	0.0009	0.0541	1.4060
(contains 6.796 per cent N).										
0.7170	0.2223	6.3659	0.0150	Extraction not made.	0.1545	0.0666	0.0140	0.0024	0.2525	6.6184
0.7550	0.2332	6.4548	0.0177		0.1062	0.0616	0.0146	0.0026	0.2027	6.7482
0.6404	0.1292	6.4690	0.0205		0.1127	0.0536	0.0150	0.0072	0.2089	6.6779
0.7067	0.0109‡	6.5319	0.0158		0.2069	0.0771	0.0167	0.0088	0.3253	6.6773
0.8514	0.1822	6.5740	0.0292§		0.0810	0.0470	0.0291	0.0102	0.1965	6.7704
0.8509	0.1556	6.6080	0.0465		0.0693	0.0530	0.0258	0.0065	0.2011	6.8091
0.6643	0.2098	6.4313	0.0489		0.0525	0.0697	0.0065	0.0044	0.1820	6.6132
0.9550	0.2347	6.8913	0.0400		0.0927	0.0726	0.0090	0.0046	0.2190	7.1102
0.7676	0.1953	6.5611	0.0292		0.1095	0.0627	0.0163	0.0058	0.2235	6.7846

ing analyses of corn.

TABLE II

	Non-protein nitrogen.				Results of Van Slyke						
	Soluble in ether.	Soluble in alcohol.	In filtrate from colloidal iron.	Total non-protein N.	Insoluble humin N.	Soluble humin N.	Acid amide N.	Arginine N.*	Cystine N.*	Histidine N.*	Lysine N.*
Alfalfa (containing 16.5% N)											
	A	B	C	D	E	F	G	H	I	J	K
	0.0152	0.0510	0.4327	0.4989	0.0968	0.1178	0.1825	0.2089	0.0243	0.1145	0.1139
	0.0152	0.0420	0.4284	0.4856	0.0970§	0.0923	0.1867	0.2119	0.0279	0.0961	0.1302
	0.0138	0.0522	0.4544	0.5204	0.0945	0.1349	0.2156	0.1977	0.0259	0.0994	0.1053
	0.0137	0.0490	0.4392	0.5019	0.0996	0.1260	0.1893	0.2220	0.0260	0.1033§	0.1165
Average	0.0145	0.0486	0.4387	0.5017	0.0970	0.1178	0.1935	0.2101	0.0260	0.1033	0.1165



concluded.

analysis.		Total non-protein + results of Van Slyke analysis.	Nitrogen lost in method of analysis.						Total.	
Amino-acid N in filtrate from bases.*	Non-amino-acid N in filtrate from bases.*		N in residue after treatment with strong NaOH.	In alcohol precipitate of hot 2 per cent $\text{CCl}_3\text{-CO}_2\text{H}$ extracts.	Unadsorbed humin (filtered from solution during decomposition of bases).	In amyl alcohol-ether extract.	In residue filtered from solution of bases.	In residue filtered from solution of filtrate from bases.	Total N lost.	Total N accounted for.

2.628 per cent N).

L	M	N	O	P	Q	R	S	T	U	V
0078	0.1015	2.4668	0.0700	Extraction not made.	0.0263	0.0160	Not determined.	0.0038	0.1160	2.5828
9902	0.0804	2.3984	0.0614		0.0292	0.0214		0.0168	0.1288	2.5272
9806	0.0314	2.4057	0.0770		0.0335	0.0079		0.0159	0.1343	2.5400
0193	0.0506	2.4547	0.0564		0.0331	0.0188		0.0099	0.1183	2.5729
9995	0.0660	2.4314	0.0662		0.0305	0.0161		0.0116	0.1244	2.5558

A word of explanation is necessary regarding these fractions. According to Van Slyke's original method (10): "During the distillation [of ammonia] all of the black coloring matter, or melanin, which is formed during the hydrolysis of the proteins, is adsorbed by the undissolved lime." The latter is filtered off, washed, and submitted to Kjeldahl analysis. The results are reported as melanin nitrogen. In the first attempt to apply the Van Slyke method to the analysis of feeds, Grindley, Joseph, and Slater (1), used the original method directly. The results for their melanin nitrogen included, therefore, any nitrogenous substances in the insoluble residue of the feed as well as the melanin or humin *formed* during the hydrolysis. Nollau (2) filtered off the insoluble residue remaining after the hydrolysis of the feed and determined by the Van Slyke method "the amino-acid content of certain commercial feedingstuffs and other sources of protein" in the filtrate. Attention was called to this fact and to the introduction of certain errors by this procedure by Grindley and Slater (3) in a second paper on "the quantitative determination of amino-acids of feedingstuff by the Van Slyke method." The nitrogen, heretofore called melanin nitrogen by them and also by Van Slyke, was called humin nitrogen in this paper.

The humin nitrogen is divided by Gortner (7) into "acid-insoluble" and "acid-soluble" (absorbed by lime) humin, the sum of the two being the total humin nitrogen. Gortner and Holm (8) report the two fractions under separate headings as insoluble humin nitrogen and soluble humin nitrogen. Other workers have reported this humin nitrogen fraction under such various headings as "humin N absorbed by lime" (Osborne, Van Slyke, Leavenworth, and Vinograd, 13), "humin nitrogen adsorbed by magnesia" (Miller, 14), and as "melanin nitrogen" divided into "a b c fraction" and "lime fraction" (Neidig and Snyder, 15). In the present paper the expressions insoluble and soluble humin have been retained. The humin nitrogen of Table III is the sum of the insoluble and the soluble humin nitrogen. It is possible that the nitrogen, or parts of it at least, in the fractions listed in Columns Q, R, S, and T of Tables I and II properly belongs to the total humin nitrogen figures but for the present it will not be included.

Here again the terminology is confusing. In Van Slyke's improved method (11) of decomposing the basic phosphotungstates by the amyl alcohol-ether method, the following statement is made:

"In some cases the aqueous and ether-amyl alcohol layers do not separate readily with a clean boundary between them. This effect is due to the presence of a slight amount of humin which may have escaped previous adsorption by calcium hydrate. In this case the unadsorbed humin is carried down with the basic phosphotungstates, and fouls the solution when their precipitate is decomposed as above described [amyl alcohol-ether method]. In order to clear the solution up, it is all, without separation of the aqueous and ether-amyl alcohol layers, passed through a Buchner funnel with suction."

Osborne, Van Slyke, Leavenworth, and Vinograd (13) in one case purified their basic phosphotungstates by reprecipitation with phosphotungstic acid and state that "all the coloring matter which accompanied the bases was extracted by the amyl alcohol and ether." The nitrogen taken up by the organic solvents is determined and reported by them as "humin N in amyl alcohol extract." Gortner and Holm (8) separated, besides the "acid-insoluble humin" and the "acid-soluble humin," a "phosphotungstic humin." This was determined by submitting the barium phosphotungstate precipitate to Kjeldahl analysis.<sup>3</sup> This fraction of humin nitrogen is undoubtedly the "humin N in amyl alcohol extract" of Osborne, Van Slyke, Leavenworth, and Vinograd and the "unadsorbed humin" of Van Slyke. This fraction is again divided by Miller (14) into "humin N insoluble in amyl alcohol" and "humin N in amyl alcohol extract."

Neidig and Snyder (15) found that a dark-colored substance usually formed along with the bases when the latter were precipitated with phosphotungstic acid. These authors refer to this substance as the "phosphotungstic humin" of Gortner and Holm. Until the present method was adopted, this dark-colored substance, which was sometimes of a sticky nature, was found repeatedly in this laboratory. With this substance present it was almost impossible to wash the basic phosphotungstates thoroughly. By the present method, outlined above, the basic

<sup>3</sup> These authors used Van Slyke's original barium hydroxide method of liberating the bases.

phosphotungstates of all four of the feeds reported here were white, gray, or slightly cream-colored granular precipitates free from any dark-colored material. However, during the decomposition of the phosphotungstates by the amyl alcohol-ether method, a scum of a dark color is formed making filtration necessary. This residue is washed carefully with ammonia-free water, amyl alcohol, and ether, and submitted to Kjeldahl analysis. The results are reported in Column Q under the heading "unadsorbed humin," the name used by Van Slyke. The clear amyl alcohol-ether extract of the phosphotungstic acid is also submitted to Kjeldahl analysis and the nitrogen content reported in Column R as the nitrogen in amyl alcohol-ether extract.

On concentrating the solution of the bases a small gray residue has been found to settle out. Van Slyke, in the original method of freeing the bases by means of barium hydrate, stated that this residue was barium phosphotungstate, which was to be filtered off and discarded. This residue has always been encountered in this laboratory even when the amyl alcohol-ether method of decomposing the basic phosphotungstates was employed. In the method used in the present work this residue is filtered off, washed carefully, and its total nitrogen determined. The results are shown in Column S. After making the filtrate from the bases up to volume (200 cc.) a residue similar to the one in the solution of the bases separates out. This is usually very small in amount, but since it has been found to be present in all cases, the nitrogen has been determined (Column T).

No attempt has been made to determine the nature of the nitrogenous constituents in these fractions. The total nitrogen has been determined in them entirely for the purpose of showing the accuracy or inaccuracy of the method as applied to the analysis of feeds.

As percentages of the total nitrogen of the feed, the humin nitrogen of oats is 5.53 per cent, of corn 3.54 per cent, of cottonseed meal 6.30 per cent, and of alfalfa 7.36 per cent.

The completeness with which the nitrogen is extracted from the finely ground feeds is shown in Column O, Table I, which gives the percentages of the total nitrogen of the feeds remaining in the residues after the last extraction with 5 per cent NaOH solution. The nitrogen in the residue from the oats, as shown by the average,

is 0.132 per cent of the original amount present; from the corn, 0.136 per cent; from the cottonseed meal, 0.430 per cent; and from the alfalfa, 2.519 per cent. Osborne and Mendel (16) found 6.0 per cent of the total nitrogen of whole corn left after extractions with 10 per cent KCl solution, 90 per cent alcohol, and 0.2 per cent KOH solution. Miller (14) in a study of the distribution of nitrogen in the alfalfa seed makes a 0.5 per cent KOH extraction and leaves between 9 and 10 per cent of the total nitrogen in the residue.

The nitrogen distribution as shown by the Van Slyke analysis includes the insoluble humin nitrogen, the soluble humin nitrogen, acid amide nitrogen, arginine nitrogen, cystine nitrogen, histidine nitrogen, lysine nitrogen, amino-acid nitrogen in the filtrate from the bases, and the non-amino-acid nitrogen in the filtrate from the basis. The question has been raised in several papers on the analysis of the proteins of feeds by the Van Slyke method that the various fractions ordinarily designated "arginine nitrogen", "histidine nitrogen," etc., may not be accurately described by these terms, on account of the heterogeneous nature of the nitrogenous constituents as well as other substances present. In the total absence of experimental evidence on this point, it seems fair to assume that the above terms are as properly applied to the fractions of nitrogen obtained in the analysis of feeds by the above described method as to the corresponding fractions obtained in the analysis of pure proteins.

It is of interest to note the variation in the basic nitrogen of the different feeds, since we know more of the requirements of animals for basic amino-acids than for any other group. The arginine nitrogen varies from about 8 per cent of the total nitrogen in alfalfa to 18.7 per cent in cottonseed meal. The cystine nitrogen is about the same in all cases but these results may, perhaps, be a little low. The histidine nitrogen is lowest in alfalfa with a value of 3.9 per cent of the total nitrogen and is highest in cottonseed meal with a value of 7.2 per cent. Lysine nitrogen is lowest in corn with a value of 2.2 per cent, slightly higher in oats with 2.8 per cent, and highest in alfalfa with 4.43 per cent. The total basic nitrogen as percentage of total nitrogen is 31.03 per cent in cottonseed meal, 21.23 per cent in oats, 17.35 per cent in alfalfa, and 16.83 per cent in corn.

In Table III the average results obtained in Table I are compared with the results on the same feeds obtained by Grindley and associates reported by Grindley (6) and by Nollau (2).

As a whole, the determinations of the different investigators do not agree well, although in some instances the agreement is quite satisfactory. The results obtained by the authors of this paper agree with those of Grindley and associates slightly better than they do with those of Nollau. The lack of concordant results is due, chiefly, to differences in methods used. Grindley and associates hydrolyzed the finely ground feeds, determined the acid amide nitrogen in the hydrolysate, filtered off the humin nitrogen, and proceeded to analyze the filtrate according to the Van Slyke method. Their results were based on the total nitrogen in the feed. Nollau removed the fat by extracting the finely ground feed with ether. The samples were then completely hydrolyzed and the insoluble humin filtered off. The total nitrogen determined in the filtrate was the basis for calculation of results of the nitrogen distribution as obtained by the Van Slyke procedure. By the improved method described above the non-protein nitrogenous constituents, most of the carbohydrates, and the fiber are removed before hydrolysis. The objectionable features of the previous methods are thereby obviated completely or at least greatly reduced. Considering the differences in procedure, Nollau's results for humin nitrogen should be lower than the results for the humin nitrogen of Grindley and associates. In general this is true. Nollau's procedure should also lead to correspondingly higher results for the remaining nitrogen values, considered on the basis of the total nitrogen of the feed. With a few exceptions this is found to be true, but the results are usually higher than this difference of procedure would warrant. The lower results for ammonia nitrogen, the amino-acid nitrogen, and non-amino-acid nitrogen in the filtrate from the bases of the method used in this work are expected because of the removal of the non-protein nitrogen. There are some differences in the results that cannot be explained on the basis of differences of procedure. For example, Nollau reports no lysine in oats while we find 2.84 per cent, he reports 8.53 per cent of lysine in corn while we find only 2.2 per cent, and again he reports no non-amino-acid nitrogen in the filtrate from the bases in corn

TABLE III.  
Comparison of Results with Those of Previous Investigators (Results Expressed as Percentage of Total Nitrogen).

	Oats.		Nollan.		Corn.		Nollan.		Cottonseed meal.		Alfalfa.	
	Hamilton, Nevens, and Grindley.	Grindley and asso- ciates, 1915.	Grindley and asso- ciates, 1915.	Hamilton, Nevens, and Grindley.	Grindley and asso- ciates, 1915.	Hamilton, Nevens, and Grindley.	Grindley and asso- ciates, 1915.	Hamilton, Nevens, and Grindley.	Grindley and asso- ciates, 1915.	Hamilton, Nevens, and Grindley.	Grindley and asso- ciates, 1915.	
Ammonia N.....	11.42	13.06	13.31	11.94	12.53	4.63	9.41	10.45	14.06	8.17	8.44	
Humin N.....	5.53	9.94	2.97	3.54	9.77	7.00	6.30	7.78	6.27	7.36	15.79	
Arginine N.....	11.65	11.42	11.42	8.73	8.49	16.19	18.71	19.52	12.77	8.00	7.68	
Cystine N.....	0.94	1.16	4.48	1.07	2.68	4.06	0.91	0.65	2.74	0.99	0.88	
Histidine N.....	5.80	4.32	9.58	4.83	3.50	4.45	7.17	5.47	7.57	3.93	7.44	
Lysine N.....	2.84	3.49	0.00	2.20	1.17	8.53	4.21	4.78	1.94	4.43	4.10	
Amino-acid N in filtrate from bases.....	42.14	51.72	43.49	46.70	52.26	49.69	40.72	42.82	45.02	38.03	44.02	
Non-amino-acid N in filtrate from bases.....	3.86	7.90	11.29	7.22	11.17	0.00	2.87	5.43	7.49	2.51	9.79	
Ether-soluble N.....	0.57			0.33			0.10			0.55		
Alcohol-soluble N.....	1.22			1.37			0.55			1.85		
Non-protein N soluble in 1 per cent CCl <sub>3</sub> CO <sub>2</sub> H—in filtrate from colloidal Fe.....	11.13			8.14			5.56			16.69		
N lost in method of analysis....	1.90			3.85			3.29			4.73		
Total.....	99.00	103.01	96.54	99.92	101.57	94.55	99.83	96.90	97.86	97.24	98.14	

while we find 7.22 per cent. The results Nollau obtained for cystine are in all cases much higher than those obtained by us.

Miller (14) in his study of the distribution of nitrogen in the alfalfa seed precipitates the protein from a 0.5 per cent KOH extract with acetic acid and analyzes the precipitate which contains only 60 per cent of the total nitrogen of the seed, according to the Van Slyke analysis. The results obtained, therefore, cannot be considered as representing the distribution of nitrogen in the entire seed. Dowell and Menaul (17) report on the "nitrogen distribution of the proteins extracted by dilute alkali from pecans, peanuts, kafir, and alfalfa." Their method, which was similar to that used by Miller, was in case of alfalfa, as follows:

"Alfalfa which was ground to pass a 40 mesh sieve was extracted with a 0.3 per cent sodium hydroxide, and 62 per cent of the nitrogen compounds were extracted. 61 per cent of the nitrogen extracted was precipitated when the solution was made slightly acid with acetic acid. The purity of the precipitated protein was found to be 85 per cent, using the factor 6.25."

Our results on alfalfa are compared with those of Dowell and Menaul, in Table IV. The agreement is obviously very poor.

TABLE IV.  
*Nitrogen Distribution of Alfalfa.*

	Dowell and Menaul.*	Hamilton, Nevens, and Grindley.†
NH <sub>3</sub> -N.....	6.8	8.17
Humin N.....	7.8	7.36
Arginine N.....	11.01	8.00
Histidine N.....	6.26	3.93
Cystine N.....	0.85	0.99
Lysine N.....	5.26	4.43
Monoamino N.....	53.53	38.03
Non-amino N.....	8.48	2.51

\* Average of two analyses; results expressed as percentage of total nitrogen of the protein preparation.

† Average of four analyses; results expressed as percentage of total nitrogen of the alfalfa.

Dowell and Menaul make the statement: "We have taken advantage of the fact that all proteins are soluble in basic solu-



tion to separate them from the other substances in foods and feeds which make it impracticable to apply the Van Slyke method to determine the nitrogen distribution." While the precipitated proteins in the case of pecans and peanuts represented a slightly larger percentage of the total nitrogen of the foodstuff, the proteins precipitated from the alkali extract of alfalfa represented less than 40 per cent of the total nitrogen of the alfalfa. Evidently a considerable fraction of the proteins of these vegetable materials is *not* soluble in basic solvents. Yet these authors conclude that "the extractions of the proteins with dilute alkaline solutions may enable us to obtain the amino-acid composition of foods and feeds by means of the Van Slyke method." With such large percentages of the nitrogen of a food or feed remaining in the unanalyzed residue, the significance of the results obtained on the extracted proteins may be seriously questioned and we doubt whether the proteins of feeds may be quantitatively separated from the other constituents by any such simple procedure as this.

#### SUMMARY AND CONCLUSIONS.

1. The amino-acid contents of oats, corn, cottonseed meal, and alfalfa, as determined by the Van Slyke method, are reported in this paper.

2. The objectionable parts of previous procedures for the application of the Van Slyke method to the determination of amino-acids of feeds have been obviated completely or at least greatly reduced by the following features of the methods used in this work: (a) The non-protein nitrogenous constituents are removed by extractions with absolute ether, cold absolute alcohol, and cold 1.0 per cent trichloroacetic acid; (b) the starch is removed by a hot 2.0 per cent trichloroacetic acid extraction; and (c) the fiber is not present during the hydrolysis of the proteins.

3. The Van Slyke method for the determination of the chemical groups characteristic of the amino-acids of proteins can be applied to the quantitative estimation of the amino-acids of feeds.

4. By further application of available methods for the estimation of other amino-acids to hydrolyzed protein solutions, prepared in a manner similar to that described for this work, it may be possible to obtain further important knowledge concerning the nutritive value of the proteins of foods and feeds.

The quantitative estimation of the amino-acids of the proteins of other common feeds is in progress at this laboratory. A study of the nitrogen distribution of the non-protein nitrogenous fraction as well as that of the humin fractions is under consideration.

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## THE SYNTHESIS OF INACTIVE PARA- AND ANTI-HYDROXYASPARTIC ACIDS (AMINOMALIC ACIDS).

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(Received for publication, June 28, 1921.)

The increasing recognition of the importance of the hydroxy-amino-acids in protein chemistry is an incentive to their more complete investigation. The particular case of hydroxyaspartic acid or aminomalic acid is of interest for several reasons. It represents the lower homologue of  $\beta$ -hydroxyglutamic acid already identified as a constituent of various proteins, and hence itself may not improbably be found among the proteins. Indeed Skraup (1) in 1904 stated that he had actually found hydroxyaspartic acid among the products of hydrolysis of casein. A careful perusal of Skraup's description of the supposedly new amino-acid does not lead to much confidence in the accuracy of the deductions drawn. In the first place the experiments are described in a fashion which makes repetition impossible; secondly no evidence is adduced that the substance in question was a dibasic acid, neither are molecular weight, optical rotation, nor a complete analysis recorded. The substance is stated to be sparingly soluble in cold water, and melts at 305–320°. A copper salt of supposedly normal constitution containing 3.5 to 4 molecules of water of crystallization, was analyzed for carbon and hydrogen, while a single nitrogen determination was made on 86 mg. of the free acid. Without further analytical or chemical evidence Skraup concludes, "Es liegt demnach bestimmt die Oxyamidobernsteinsäure vor, die bisher überhaupt noch nicht beschreiben worden ist." Experiments on the synthesis of the acid were stated to be in progress but have not been reported.

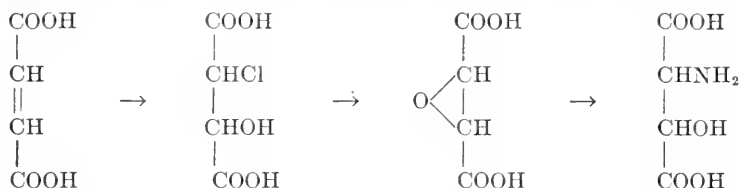
In the same year Erlenmeyer (2) wrote, "Wie im experimentellen Theile gezeigt werden wird, ist es mir gelungen . . . von Oxalylhippursäureester aus zur Amidoäpfelsäure, welche

kurzlich von Skraup als Spaltungsprodukt des Caseins nachgewiesen wurde, zu gelangen." But a careful examination of the experimental part of this and other papers by Erlenmeyer has failed to disclose any further reference to the acid.

Almost simultaneously Neuberg and Silbermann (3) announced another synthesis of the acid by the limited action of nitrous acid upon mesodiaminosuccinic acid. A small amount of a readily soluble copper salt (1.2 gm.) was obtained which apparently contained no water of crystallization and which gave an analysis agreeing excellently with that calculated for the normal salt. The free acid was not obtained in sufficient amount for analysis but its melting point is given as 314–318°, in substantial agreement with Skraup's observation. As a matter of fact, as will appear in the experimental portion of the paper, hydroxyaspartic acid does not give a soluble copper salt under the conditions employed by Neuberg and Silbermann; namely, boiling with copper carbonate. Almost the whole of the acid remains as a light blue very sparingly soluble copper salt, containing water of crystallization, and the salt has an abnormal composition containing three equivalents of copper. Furthermore, the acid does not melt at the temperature given by either Skraup or by Neuberg and Silbermann but begins to decompose slowly above about 230° but is not melted even at 360°. The writer believes that Neuberg and Silbermann's compound contained no hydroxyaspartic acid and the apparent correspondence of the analysis of their copper salt with that calculated for the normal compound and the agreement of the melting point with Skraup's observation remains unexplained.

On the other hand experiments made on a small preliminary scale by Lossen (4) in 1906, while described by the author as incomplete, indicated that an acid,  $C_2H_2 \cdot (OH \cdot NH_2)(COOH)_2 + H_2O$ , presumably an aminomalic acid, was formed by the action of ammonia on fumarylglucydic acid. Neither the free acid nor barium salt described by Lossen agree in detail with the products obtained by the writer, but there is no question that much aminomalic or hydroxyaspartic acid was present in Lossen's substances. In the experimental part of the paper details are given showing clearly the difficulty of satisfactorily crystallizing the products from the above reaction.

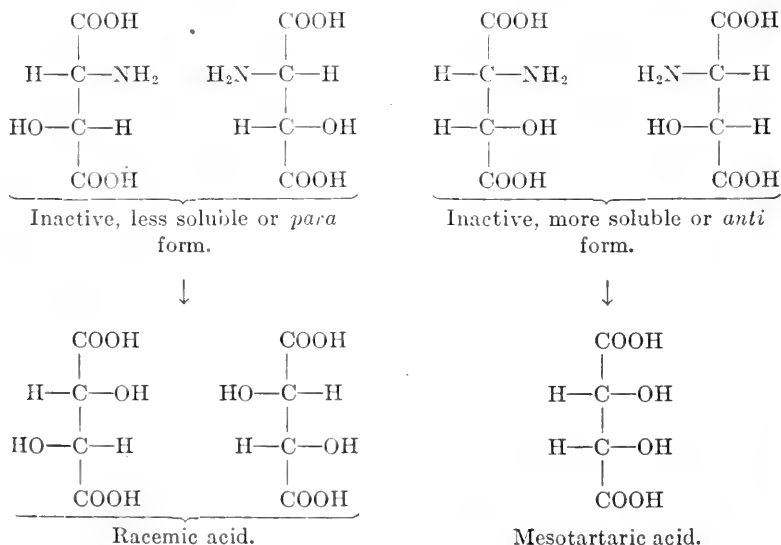
The synthesis made use of in the present investigation is based on the action of ammonia on chloromalic acid, prepared by the action of chlorine on sodium fumarate. The action of ammonia on chloromalic acid first results in the production of fumaryl-glycidic acid so that the reaction is essentially similar to that used by Lossen. The changes may be represented as follows:



Chloromalic acid reacts in the cold with strong aqueous ammonia but even after 2 weeks the conversion into hydroxyaspartic acid is incomplete and the products are difficult to separate. On the other hand hydroxyaspartic acid is not completely stable in aqueous solution even at 125° and in alkaline or acid solution it is still more readily decomposed. On the whole it was found that the reaction was best carried out by heating the chloromalic acid with five parts of concentrated aqueous ammonia for about 10 hours in an iron autoclave immersed in a boiling water bath. On isolating the amino-acid as described in the experimental part of this paper, or by many other methods which need not be described, the product was obtained in the form of a viscous mass which became friable on treatment with glacial acetic acid or with alcohol and which was extraordinarily soluble in water. The purified product analyzed satisfactorily for  $\text{C}_4\text{H}_7\text{O}_5\text{N}$  and its molecular weight as determined by Barger's method or by titration was close to 149. The whole of the nitrogen was in the amino form and could be liberated with nitrous acid. Early efforts at crystallization failed, but eventually it was found possible to obtain well formed crystals which separated slowly from aqueous solutions of moderate concentration. Once in possession of crystals for seeding purposes it was found very easy to crystallize new preparations. On successive fractional crystallization it was found possible to resolve the substance into two isomeric forms with similar chemical properties but differing in solubility and crystalline form. The least soluble form which

is present in smaller amount than the other is very sparingly soluble in cold water when pure. On decomposition with nitrous acid it gives chiefly, if not exclusively, racemic acid. The more soluble form crystallizes slowly and has a great tendency to form supersaturated solutions, but even this isomer requires about thirty parts of cold water to dissolve it. The effect on the solubility of the one form caused by the presence of the other is remarkable. The more soluble form on treatment with nitrous acid gives mesotartaric acid. The two forms are interconvertible to a certain extent and in particular the less soluble form is produced on heating a 25 per cent aqueous solution of its isomer for several hours at 120–125°.

From the preceding facts it is clear that two optically inactive stereoisomeric forms of hydroxyaspartic acid exist corresponding in structure to mesotartaric and racemic acids. Each of these two forms should be separable into active components giving a total of four active and two inactive forms. The resolution into active components is as yet incomplete, but will be reported shortly. The various isomeric forms of hydroxyaspartic acid in relation to mesotartaric and racemic acids are shown in the subjoined formulas:

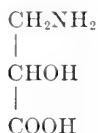


Hydroxyaspartic acid thus furnishes a good example of the occurrence of two inactive resolvable isomers of a compound containing two dissimilar asymmetric carbon atoms. The example gains in interest partly from the fact that hydroxyaspartic acid represents the simplest known case of this type of isomerism and also on account of the direct relationship with the classical isomerism of the tartaric acids.

The separate designation of the two inactive hydroxyaspartic acids offers considerable difficulties such as are often encountered in other asymmetrical derivatives of succinic acid. The use of *cis* and *trans* for differentiating such saturated compounds is generally agreed to be undesirable. The use of the term "*fumaroid*" for the less soluble and higher melting isomer and "*maleinoid*" for the more soluble and lower melting one seems inappropriate in the present case although the fumaroid form of hydroxyaspartic acid would be the one related to mesotartaric acid and as is well known fumaric acid is directly oxidizable to racemic and not mesotartaric acid. The use of the term "*allo*" for a second isomer is convenient in cases such as isoleucine when one form can be appropriately assigned the simpler name without a prefix. On the whole the use of the terms "*para*" and "*anti*" already made use of by Bischoff for isomeric alkylsuccinic acids appears least open to objection. In the present case the differentiation is not made on the basis of assigning the "*para*" prefix to the higher melting isomer since neither form possesses a definite melting point. Rather, the term "*para*" is assigned to the less soluble form which is convertible into *para*-tartaric or racemic acid while the more soluble form is designated "*anti*" since it gives *anti*- or mesotartaric acid on replacement of its amino group by hydroxyl.

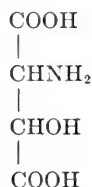
The *anti* and *para* forms of hydroxyaspartic acid are readily differentiated by the unaided eye after a little experience and still more readily under the microscope. It is hoped to obtain a good crystallographic description of the two forms. Both forms give an intense pyrrole reaction on gentle heating. Most of the salts are similar though the acid calcium salts show differences which are to be referred to later. The phenylhydantoin derivative of the *anti* acid is much more readily soluble than the *para* compound. Reference must be made to the copper and zinc salts

both of which are of abnormal composition. Both *para*- and *anti*-hydroxyaspartic acids, when dissolved in two equivalents of sodium hydroxide, give sparingly soluble precipitates with neutral copper or zinc acetates, the reaction of the solution becoming acid. Analyses of the salts show the presence of three equivalents of metal, indicating that the hydrogen of the hydroxyl group has been replaced by metal. A similar phenomenon is well known to occur with tartrates and malates and Fischer and Leuchs (5) has found that the copper salt of isoserine is similarly abnormal. The relationship of isoserine and hydroxyaspartic acid is obviously a close one.



Isoserine.

Salt contains 2 equivalents of Cu.



Hydroxyaspartic acid.

Salts contain 3 equivalents of Cu or Zn.

Hydroxyaspartic acid prevents the precipitation of iron and copper by excess of alkali as is the case with the analogous tartrates and malates.

Finally a word may be said in regard to the possible presence of hydroxyaspartic acid in proteins. The present situation appears to be that the acid certainly has not yet been identified and Skraup's assertions to the contrary are undoubtedly ill founded. On the other hand, certain newer experiments made by the writer have given negative results, but it is not at all certain that the methods used were adequate. The problem is a difficult one and needs further investigation.

Experiments on the preparation of hydroxyaspartic acid ethyl esters, using ethyl alcohol and hydrogen chloride, indicated their formation in good yield. The esters were liberated from the hydrochlorides by means of potassium carbonate and extracted with ether. On attempting to distil the esters under moderately low pressure (10 mm.), a great deal of decomposition occurred and only very little impure ester, boiling at about



140°, was obtained. It appears improbable that the ester method would be useful for the detection of hydroxyaspartic acid in proteins.

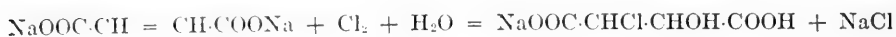
Hydroxyaspartic acid in both *anti* and *para* forms reduces potassium permanganate slowly in acid, neutral, or alkaline solution. On oxidation of the neutral salts with sodium hypochlorite or chloramine-T, a good deal of glyoxal is formed together with some tartronic semialdehyde. Neither form gives Fenton's reaction for tartaric acid with hydrogen peroxide and ferrous sulfate, though they give various color reactions with phenols and sulfuric acid resembling more or less those given by malic and tartaric acids.

On heating the *anti* form with strong aqueous potash it was completely decomposed, and on heating with a little hydrochloric acid at 125° it was also found to be unstable. On heating it with water at 125° for 4 hours a partial conversion into the *para* form was accomplished. The reverse change could also be demonstrated though less readily; namely, the formation of the *anti* from the *para* acid.

#### EXPERIMENTAL PART.

*Chloromalic Acid*.—The following modification of Lossen's method has proved useful for the preparation of considerable quantities of chloromalic acid. A large bottle (10 to 20 liters) of approximately known capacity is filled with water and inverted in a trough. Chlorine gas from a cylinder of liquid chlorine is rapidly passed in until the bottle is filled when it is removed and corked. The weight of chlorine in the bottle is then calculated. An amount of fumaric or maleic acid equivalent to the chlorine is then weighed out, suspended in water, and neutralized with sodium hydroxide, using phenolphthalein as indicator. The sodium fumarate is diluted with ice water to about 1 per cent concentration and a few cc. are rapidly poured into the bottle of chlorine which is then closed with a stopper carrying a rubber tube dipping into the bulk of the fumarate solution. On agitating the gas bottle, absorption of the chlorine rapidly occurs and the whole of the solution is readily sucked over. The mixture is allowed to stand until the following morning when it

is neutralized with sodium hydroxide, an amount of alkali being required which is exactly half of that used originally to neutralize the fumaric acid.



A 20 liter gas bottle serves for quantities of fumaric acid in the neighborhood of 110 gm.; its use will be found far more convenient than the use of strong chlorine water described by Lossen. Crystallized barium chloride ( $2\frac{1}{2}$  equivalents) is then added to the neutralized chloromaleate solution and the whole vigorously shaken for half an hour on a machine so as to obtain a readily filterable precipitate. After a few hours standing the barium chloromaleate is filtered off, washed with water, and dried carefully in a warm place as it retains water mechanically with unusual persistence. The air-dry barium salt is crushed, weighed, and then transferred to a thick walled bottle and covered with ether. Concentrated hydrochloric acid is then added by degrees with shaking and cooling until about a 10 per cent excess has been added. The ether layer is decanted from the aqueous barium chloride suspension and the extraction repeated with fresh ether four or five times. The combined ether extracts are washed with a few drops of water and then evaporated at a low temperature. Chloromaleic acid remains as an oil which quickly solidifies to an opaque crystalline mass of the pure substance. The yield averages 45 to 50 per cent of the theoretical amount calculated from the fumaric acid used.

*Fumarylglucidic Acid.*—The filtrate from the barium chloromaleate still contains a good deal of dissolved salt and this may be converted into the less soluble barium salt of fumarylglucidic acid by adding an additional equivalent of sodium hydroxide and allowing the mixture to stand for a further 24 hours. The barium salt thus obtained as described by Lossen is filtered off and dried. Instead of using Lossen's method of liberating the free acid which has not given good results in the writer's hands it is better to decompose the salt at  $0^\circ$  with concentrated hydrochloric acid in a calculated amount as already described for barium chloromaleate, but using ethyl acetate as solvent instead of ether. The ethyl acetate is removed by distillation under reduced pressure

when fumarylglucidic acid readily crystallizes out in amount equivalent to 20 to 25 per cent of the fumaric acid originally employed. Fumarylglucidic acid thus obtained serves equally well as chloromalic acid for the preparation of hydroxyaspartic acid.

*Hydroxyaspartic Acids.*—Chloromalic acid (56 gm.) is added by degrees to 300 cc. of concentrated aqueous ammonia contained in a porcelain beaker surrounded by an ice bath. A little ether may be added with advantage to promote the dissolution of the acid. Ammonia gas is then passed in rapidly until the mixture approaches saturation. The covered beaker is then placed in an iron autoclave suspended in a water bath which is brought to the boiling point for about 10 hours. If the reaction is carried out in iron tubes without other protection a good deal of iron remains dissolved in the clear solution and is decidedly difficult to remove quantitatively. The use of the porcelain beaker is therefore strongly recommended. After heating, the contents of the beaker are diluted with an equal volume of water and slightly more than two equivalents (28 gm. instead of 26.6 gm.) of sodium hydroxide are added. The solution is then evaporated to a small volume under reduced pressure so as to remove most of the ammonia. The residue is taken up in water and made fairly strongly acid to Congo red with nitric acid. Silver nitrate is then added in slight excess over that needed to remove the whole of the chloride. The filtrate is diluted to about 750 cc. and a concentrated solution of 150 gm. crystallized lead acetate is added. The solution is then incompletely neutralized by the addition with stirring of either dilute sodium hydroxide or ammonia leaving the supernatant fluid still distinctly acid with the liberated acetic acid. The lead precipitate is thoroughly washed with cold water, transferred to a flask, suspended in water, and decomposed with hydrogen sulfide under slight pressure in the customary fashion. After complete decomposition the mixture is heated on the water bath, and filtered from lead sulfide, and concentrated under diminished pressure. A syrup is finally obtained which may be conveniently purified by stirring it successively with glacial acetic acid and methyl alcohol. A white sticky mass results which after washing with alcohol quickly becomes brittle and dusty in the desiccator. The product represents almost

entirely a mixture of the two forms of hydroxyaspartic acid, as is shown by the following analyses. The substance was dried *in vacuo* over phosphorus pentoxide at 60°.

Prep. I.	0.1180 gm. substance:	0.1400 gm. CO <sub>2</sub> ,	0.0499 gm. H <sub>2</sub> O.
Prep. II.	0.2132 " "	:0.2541 " CO <sub>2</sub> ,	0.0950 " H <sub>2</sub> O.
	0.2052 " "	:0.0191 " N (Kjeldahl).	
	0.0106 " "	:1.76 cc. N, 18°; 754 mm. (Van Slyke).	
C <sub>4</sub> H <sub>7</sub> O <sub>5</sub> N.	Calculated.	C 32.2,	H 4.7, N 9.4.
	Found.	C 32.3, 32.7, H 4.7, 4.95,	N 9.31, 9.46.

On titration 1.850 gm. required 12.7 cc. normal sodium hydroxide to neutralize, using litmus paper as indicator. This is equivalent to a molecular weight of 146, compared with a calculated value of 149. On employing Barger's micro method for molecular weight determinations it was found that an aqueous solution of the acid containing 2.78 per cent was in equilibrium with an 0.18 normal solution of tartaric acid indicating a molecular weight of 154. Silver and barium salts on analysis also gave results in close accord with the preceding figures.

With regard to the separation of hydroxyaspartic acids it may be mentioned that the silver or mercury salts may be used to replace the use of lead, but they offer no significant advantage. The product is also readily precipitated by excess of barium hydroxide as the neutral barium salt but curiously enough it is almost impossible in the writer's experience to recover the acid satisfactorily by decomposition with sulfuric acid since a point is soon reached when addition of either barium hydroxide or sulfuric acid or both fails to produce a precipitate of barium sulfate and the product is heavily contaminated with inorganic substances.

*Separation of Para- and Anti-Hydroxyaspartic Acids.*—Thus far simple fractional crystallization from water is the only method employed for the separation of the two isomers, although it is possible that the differences in solubility of the acid calcium salts may eventually furnish a more convenient method. It is a curious fact that while the *para* and *anti* forms are relatively sparingly soluble when once separated, the mixture of acids separated as described in the preceding section retains its extreme solubility in water apparently indefinitely. In order to separate the two acids it is convenient to dissolve the mixed product in

about five parts of hot water and on cooling, either expose to the air or better, seed the mixture with a few crystals of the *para* acid obtained from a previous operation. Crystallization is slow, but on standing over night a fair crop of crystals usually accumulates, chiefly made up of the *para* form. The *para* form is easily identified by the fact that its crystals are usually small, white, and opaque, somewhat resembling aspartic acid, whereas the *anti* form crystallizes into large stout crystals, often a couple of centimeters long, which are transparent while moist and only become opaque on drying. Under the microscope the *para* acid appears to crystallize in small cubes or plates while the *anti* form crystallizes in hexagonal plates and thick prisms of complicated form.

The first crop of crystals containing excess of the *anti* form is most conveniently purified by washing it with a little warm water in which the *anti* acid dissolves much more quickly, and then recrystallizing the residue once or twice from boiling water until it appears perfectly homogeneous.

The *anti* form crystallizes slowly from the first mother liquor and care should be taken that the solution should not become too concentrated. The crystals are best removed from time to time and finally the accumulated product should be rapidly dissolved in hot water, rejecting any sparingly soluble and slowly dissolving *para* acid which may be identified with the microscope. Crystallization is allowed to take place slowly at room temperature. The yield of *para* acid seldom exceeds 20 per cent of the mixed product while the amount of *anti* form varies considerably but always greatly exceeds the other. In well conducted experiments as much as 60 to 70 per cent of the crude product may be obtained in the *anti* form while often much remains in the mother liquors in a difficultly recoverable condition. Occasionally a product is encountered, crystallizing in well formed needles and giving at first the appearance of homogeneity, but on slow recrystallization from water it will be found to be a mixture of the two forms already dissolved.

*Para-Hydroxyaspartic Acid*.—The purified acid, separated as described in the preceding section, was recrystallized from water and obtained in the form of small opaque cubic crystals. It has no sharp melting point but on heating it slowly decomposes above

235° and gives a solid mass which does not melt even at 350°. On ignition it gives vapors which furnish a strong pyrrole reaction with the pine-splinter test. The acid dissolves in about 300 parts of cold water, but in the presence of isomerides or other impurities its apparent solubility is enormously increased.

Analysis. 0.1941 gm. substance:0.2278 gm. CO<sub>2</sub>, 0.0872 gm. H<sub>2</sub>O.  
 0.0155 " " :2.58 cc. N, 20°, 756 mm. (Van Slyke).  
 C<sub>4</sub>H<sub>7</sub>O<sub>5</sub>N. Calculated. C 32.2, H 4.70, N 9.40.  
 Found. C 32.0, H 4.99, N 9.48.

*Phenylhydantoin Derivative of Para-Hydroxyaspartic Acid.*—1 gm. of the acid was dissolved in 15 cc. normal sodium hydroxide and an excess of phenylisocyanate (1 gm.) was added by degrees with frequent shaking. After the cyanate had been completely decomposed the mixture was filtered from diphenylurea and the filtrate acidified with concentrated hydrochloric acid (3 cc.). No separation occurred, so the solution was evaporated on the water bath to small bulk. A separation of the hydantoin occurred at this stage, but as it is apt to be contaminated with sodium chloride it was convenient to separate the bulk of the latter by taking the hydantoin up in methyl alcohol, filtering, and concentrating. On cooling the hydantoin quickly separated out and was spread on porous tile and then recrystallized from 50 per cent methyl alcohol in which it is freely soluble. It crystallizes in bundles of fine white needles and melts sharply at 201.5–202.5° (uncorrected). The substance while freely soluble in water is less soluble in water, alcohol, and acetone than the corresponding derivative of the *anti* acid. It is very slightly soluble in chloroform.

Analysis. 0.1500 gm. substance:0.2894 gm. CO<sub>2</sub>, 0.0564 gm. H<sub>2</sub>O.  
 C<sub>11</sub>H<sub>10</sub>N<sub>2</sub>O<sub>5</sub>. Calculated. C 53.1, H 4.00.  
 Found. C 52.6, H 4.18.

The salts of the two acids in most particulars resemble each other closely as regards properties and composition. The salts of the alkali metals and of magnesium are freely soluble in water while those of the heavy metals are all sparingly soluble. One noteworthy difference in the salts of the two acids is the fact that the acid calcium and barium salts of the *para* acid are decidedly

less soluble than those of the *anti* acid and unlike the latter do not melt in boiling water.

*Acid Calcium Salt of Para-Hydroxyaspartic Acid.*—The salt was obtained by adding clear lime water until a suspension of the *para* acid in hot water was just neutral to litmus. On concentrating slightly, fine glistening quadrilateral plates readily separate. The same salt is obtained by adding calcium acetate to a hot saturated solution of the acid. Occasionally the crystals appear as coffin-shaped plates. The substance is highly characteristic and the air-dried salt contains 5 molecules of water of crystallization which are given off at  $120^{\circ}$  *in vacuo*.

Analysis. 0.1295 gm. substance:0.0281 gm.  $\text{H}_2\text{O}$ , 0.0415 gm.  $\text{CaSO}_4$ .  
 $(\text{C}_4\text{H}_6\text{O}_5\text{N})_2\text{Ca}\cdot 5\text{H}_2\text{O}$ . Calculated.  $\text{H}_2\text{O}$  21.1, Ca 9.37.  
Found.  $\text{H}_2\text{O}$  21.7, Ca 9.44.

*Neutral Calcium Salt (Para).*—On dissolving one equivalent of the acid in two equivalents of ammonia or sodium hydroxide solution, and then adding calcium acetate, the neutral calcium salt is obtained as a white sparingly soluble granular precipitate. It contains some water of crystallization which is removed at  $120^{\circ}$  *in vacuo*. The dry salt was found to contain 21.3 per cent calcium, the calculated value being 21.4.

*Acid Barium Salt (Para).*—This salt was prepared in the same fashion as the acid calcium salt by adding barium hydroxide to a hot solution of the acid. It is important to have the final reaction of the solution very slightly acid to litmus as otherwise the salt is contaminated with some of the sparingly soluble neutral salt. The acid barium salt is moderately soluble even in cold water and readily soluble in hot water. When it separates rapidly it forms a granular powder, but when crystallized slowly it gives well formed plates, containing 3 molecules of water of crystallization which it loses completely at  $135^{\circ}$  *in vacuo*.

Analysis. 0.3025 gm. substance:0.0235 gm.  $\text{H}_2\text{O}$ , 0.1415 gm.  $\text{BaSO}_4$ .  
 $(\text{C}_4\text{H}_6\text{O}_5\text{N})_2\text{Ba}\cdot 3\text{H}_2\text{O}$ . Calculated.  $\text{H}_2\text{O}$  11.1, Ba 28.1.  
Found.  $\text{H}_2\text{O}$  11.1, Ba 27.5.

*Neutral Barium Salt (Para).*—The salt is most easily obtained by adding a hot aqueous solution of the acid to an excess of barium hydroxide solution. An immediate precipitation of a

very insoluble barium salt takes place and the precipitate is at once filtered off, washed with water, and dried. It appears to be stable and anhydrous.

Analysis. 0.2618 gm. substance:0.2156 gm.  $\text{BaSO}_4$ .

$(\text{C}_4\text{H}_5\text{O}_5\text{N})\text{Ba}$ . Calculated. Ba 48.2.

Found. Ba 48.4.

*Copper Salt (Para).*—The only copper salt obtained in a pure condition is the very sparingly soluble compound which contains three equivalents of copper to one of the acid and hence is an abnormal salt. It is best obtained by neutralizing the *para* acid with two equivalents of sodium hydroxide and then adding a moderate excess of neutral copper acetate. The reaction of the medium turns acid and a voluminous light blue powder is precipitated. The product is soluble in excess of alkali giving a product resembling Fehling's solution and is also soluble in a large excess of copper acetate. The composition of the air-dried product varies slightly but most closely accords with the following formula:  $(\text{C}_4\text{H}_4\text{O}_5\text{N})_2\text{Cu}_3 \cdot 8\text{H}_2\text{O}$ . The exact determination of the combined water is somewhat difficult and is best carried out under greatly diminished pressure over phosphorus pentoxide at a temperature of about  $135^\circ$ . The extremes and average of a number of analyses are given below:

$(\text{C}_4\text{H}_4\text{O}_5\text{N})_2\text{Cu}_3 \cdot 8\text{H}_2\text{O}$ . Calculated.	$\text{H}_2\text{O}$ 22.9, Cu 30.4, N 4.47, C 15.3.
Found.	$\text{H}_2\text{O}$ 20.5–24.2, Average 22.7.
	Cu 29.6–31.1, Average 30.3, N 4.48.
	C 15.6.

*Zinc Salt (Para).*—The zinc salt was obtained in precisely the same way as that employed for the copper salt, substituting zinc acetate for copper acetate. The reaction of the solution becomes acid and the preprecipitated white sparingly soluble salt contains three equivalents of zinc to one of acid. The analysis indicates  $(\text{C}_4\text{H}_4\text{O}_5\text{N})_2\text{Zn}_3 \cdot 7\text{H}_2\text{O}$  as the most probable formula for the air-dried salt. The combined water was determined by drying over phosphorus pentoxide at 2 mm. pressure at  $125^\circ$ .

Analysis. 0.2165 gm. substance:0.0432 gm.  $\text{H}_2\text{O}$ , 0.0862 gm.  $\text{ZnO}$ .

0.0210 " " :1.70 cc. N,  $21^\circ$ , 760 mm. (Van Slyke).

$(\text{C}_4\text{H}_4\text{NO}_5)_2\text{Zn}_3 \cdot 7\text{H}_2\text{O}$ . Calculated.  $\text{H}_2\text{O}$  20.5, Zn 31.9, N 4.56.

Found.  $\text{H}_2\text{O}$  20.0, Zn 32.1, N 4.58.



*Silver Lead and Mercury Salts (Para).*—Most of the heavy metals produce sparingly soluble precipitates with hydroxyaspartic acid or its salts. The composition of the lead and mercury salts varies according to the conditions of their formation, from the acid salts, to neutral and basic salts. Some of these have been analyzed but it is not easy to secure perfectly uniform products. The silver salt on the other hand has a constant normal composition. It is obtained as a white curdy insoluble precipitate on adding silver nitrate to *para*-hydroxyaspartic acid, neutralized with two equivalents of ammonia or sodium hydroxide. It is not particularly sensitive to light.

Analysis. 0.2920 gm. substance:0.1747 gm. Ag.  
 $C_4H_5O_5NaAg_2$ . Calculated. Ag 59.5.  
 Found. Ag 59.8.

*Anti-Hydroxyaspartic Acid.*—The more soluble acid, separated as previously described, was recrystallized repeatedly from water. It is freely soluble in hot water but requires about thirty-three parts of cold water (18°) for solution. As already stated its apparent solubility is enormously increased by the presence of its isomer or other impurity and it shows considerable tendency to the formation of supersaturated solutions.

Analysis. 0.1501 gm. substance:0.1761 gm.  $CO_2$ , 0.0607 gm.  $H_2O$ .  
 0.0150 " " :2.54 cc. N, 20°, 756 mm. (Van Slyke).  
 $C_4H_7O_5N$ . Calculated. C 32.2, H 4.70, N 9.40.  
 Found. C 32.0, H 4.50, N 9.58.

The molecular weight determined by titration, using litmus paper as indicator, was found to be 147, compared with a calculated value of 149 (0.300 gm. required 10.2 cc. of 0.2 N sodium hydroxide). Using Barger's capillary tube method a 2.622 per cent solution was found equivalent to 0.18 normal tartaric acid, giving a value of 146 for molecular weight.

*Phenylhydantoin Derivative of Anti-Hydroxyaspartic Acid.*—The reaction was carried out as described for the corresponding *para* compound. The methyl alcohol extract crystallized rather less readily and was too soluble to permit of convenient crystallization from water or alcohol. Crystallization was most readily effected by dissolving the compound in hot acetone in which

it is easily soluble and then adding two volumes of chloroform. The hydantoin crystallizes readily in nacreous plates suggestive of leucine in appearance. It melts sharply at 196–198° uncorrected, and is sparingly soluble in chloroform or ethyl acetate.

Analysis. 0.1825 gm. substance: 0.3545 gm. CO<sub>2</sub>, 0.0721 gm. H<sub>2</sub>O.

C<sub>11</sub>H<sub>10</sub>N<sub>2</sub>O<sub>5</sub>. Calculated. C 52.8, H 4.00.

Found. C 52.9, H 4.30.

The following salts of *anti*-hydroxyaspartic acid were all prepared as described for those of the *para* acid, hence only the analyses and any points of difference between the two series are recorded below.

*Acid Calcium Salt (Anti)*.—The salt differs from the *para* compound in that it melts to a gum in boiling water. It is freely soluble and is best obtained by evaporation of its aqueous solution at room temperature in a desiccator. It contains 4 molecules of water of crystallization, and easily passes over into the neutral salt.

Analysis. 0.1514 gm. substance: 0.0260 gm. H<sub>2</sub>O, 0.0395 gm. CaCO<sub>3</sub>.

0.0150 “ “ : 1.65 cc. N, 21°, 760 mm. (Van Slyke).

(C<sub>4</sub>H<sub>6</sub>NO<sub>5</sub>)<sub>2</sub>Ca·4H<sub>2</sub>O. Calculated. H<sub>2</sub>O 17.7, Ca 9.80, N 6.30.

Found. H<sub>2</sub>O 17.2, Ca 9.80, N 6.23.

*Neutral Calcium Salt (Anti)*.—The salt is a white granular sparingly soluble substance containing close to 2 molecules of water of crystallization.

Analysis. 0.1521 gm. substance: 0.0243 gm. H<sub>2</sub>O, 0.0677 gm. CaCO<sub>3</sub>.

C<sub>4</sub>H<sub>5</sub>N<sub>2</sub>O<sub>5</sub>Ca·2H<sub>2</sub>O. Calculated. H<sub>2</sub>O 16.1, Ca 18.0.

Found. H<sub>2</sub>O 16.0, Ca 17.8.

*Acid Barium Salt (Anti)*.—Melts with some difficulty in boiling water and separates from cool solutions in crystalline nodules. It dissolves in about twenty-five parts of cold water and contains 3 molecules of water of crystallization which are removed at 105°.

Analysis. 0.2383 gm. substance: 0.0278 gm. H<sub>2</sub>O, 0.1097 gm. BaSO<sub>4</sub>.

(C<sub>4</sub>H<sub>6</sub>O<sub>5</sub>N)<sub>2</sub>Ba·3H<sub>2</sub>O. Calculated. H<sub>2</sub>O 11.1, Ba 28.1.

Found. H<sub>2</sub>O 11.6, Ba 27.1.

*Neutral Barium Salt (Anti).*—A very sparingly soluble granular powder containing no water of crystallization, closely resembling the *para* salts.

Analysis. 0.1606 gm. substance:0.1307 gm.  $\text{BaSO}_4$ .  
 $(\text{C}_4\text{H}_5\text{O}_5\text{N})\text{Ba}$ . Calculated. Ba 48.2.  
 Found. Ba 48.1.

*Copper Salt (Anti).*—In appearance and properties as well as composition the copper salt of the *anti* acid closely resembles that of the *para* acid.

Analysis. 0.1788 gm. substance:0.0393 gm.  $\text{H}_2\text{O}$ , 0.0679 gm.  $\text{CuO}$ .  
 0.0300 “ “ :2.32 cc. N,  $20^\circ$ , 762 mm. (Van Slyke).  
 $(\text{C}_4\text{H}_4\text{O}_5\text{N})_2\text{Cu}_3 \cdot 8\text{H}_2\text{O}$ . Calculated.  $\text{H}_2\text{O}$  22.9, Cu 30.4, N 4.47  
 Found.  $\text{H}_2\text{O}$  22.0, Cu 30.4, N 4.31

*Zinc Salt (Anti).*—This salt closely resembles the *para* salt. It is sparingly soluble and contains three equivalents of zinc to one of the acid.

Analysis. 0.1984 gm. substance:0.0397 gm.  $\text{H}_2\text{O}$ , 0.0782 gm.  $\text{ZnO}$ .  
 $(\text{C}_4\text{H}_4\text{O}_5\text{N})_2\text{Zn}_3 \cdot 7\text{H}_2\text{O}$ . Calculated.  $\text{H}_2\text{O}$  20.5, Zn 31.9.  
 Found.  $\text{H}_2\text{O}$  20.0, Zn 31.7.

*Silver and Lead Salts (Anti).*—The silver salt was obtained as a white insoluble curdy precipitate containing 59.7 per cent of silver (calculated 59.8). The normal lead salt is obtained as a dense white heavy precipitate on adding lead acetate to a neutral solution of the sodium or ammonium salt of the *anti* acid. It contained no water of crystallization and gave on analysis 58.1 per cent of lead (calculated 58.3). On adding lead acetate to a solution of the free acid a microcrystalline insoluble salt of complex composition (48.3 per cent lead) separates out, and on washing with water is slowly converted into the normal salt.

*Action of Nitrous Acid on Anti- and Para-Hydroxyaspartic Acids.*—In each case the *anti* or *para* acid (1.49 gm.) was dissolved in 150 cc. of water and 3 cc. of concentrated hydrochloric acid. The solutions were kept at room temperature and sodium nitrite (1 gm.) was added by degrees in the course of a day. Nitrogen was freely evolved on agitating the solutions. The following day the solutions were almost neutralized with ammonia

using litmus as indicator, and an excess of calcium acetate was added. The precipitated calcium salts which separated rather slowly were in each case filtered off, dissolved in a few drops of hydrochloric acid, and reprecipitated with ammonia. The *anti*-hydroxyaspartic acid gave a granular calcium salt, composed of small prisms and contained close to 3 molecules of water of crystallization, which it lost at 170° and appeared identical in every respect with calcium mesotartrate prepared for comparison.

Analysis. 0.1463 gm. substance:0.0331 gm. H<sub>2</sub>O, 0.0789 gm. CaSO<sub>4</sub>.

C<sub>4</sub>H<sub>4</sub>O<sub>6</sub>Ca·3H<sub>2</sub>O. Calculated. H<sub>2</sub>O 22.3, Ca 16.5.

Found. H<sub>2</sub>O 22.5, Ca 16.0.

The *para*-hydroxyaspartic acid gave a calcium salt which was slightly more soluble than that from the *anti* acid and was made up of needles. Few if any of the mesotartrate prisms were found on microscopic examination. The salt contained close to 4 molecules of water which were removed at 170° and appeared identical with calcium racemate prepared for comparison.

Analysis. 0.1838 gm. substance:0.0498 gm. H<sub>2</sub>O, 0.0989 gm. CaSO<sub>4</sub>.

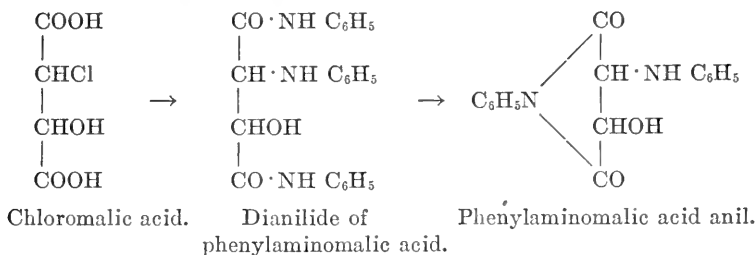
C<sub>4</sub>H<sub>4</sub>O<sub>6</sub>Ca·4H<sub>2</sub>O. Calculated. H<sub>2</sub>O 27.7, Ca 15.4.

Found. H<sub>2</sub>O 27.1, Ca 15.8.

The yield of mesotartaric and racemic acids was only equivalent to about 25 per cent of the theoretical amount.

*Action of Aniline on Chloromalic Acid.*—In connection with the study of other bases than ammonia upon chloromalic acid, the following two derivatives were obtained by the action of aniline. Chloromalic acid (1 mol) with aniline (4 mols) were heated for 3 hours in a flask placed in a paraffin bath at 130°. The sticky mass was well washed with dilute hydrochloric acid to remove excess of aniline and then heated with alcohol. A small amount of sparingly soluble substance which proved to be an "anil" derivative of phenylaminomalic acid was filtered off and subsequently recrystallized from glacial acetic acid in which it is readily soluble when hot but sparingly soluble at room temperature. It crystallizes in bright yellow plates and melts at 238–239° (uncorrected). From the alcoholic filtrate a dianilide of phenylaminomalic acid was obtained which was recrystallized from 90 per cent methyl alcohol. It crystallizes in nodular clumps

of bright yellow needles and on heating softens above 200° and melts at 210–211° (uncorrected). The yield of the latter substance is considerably greater than that of the "anil". Its reaction may be represented as follows:



Analyses. 0.1102 gm. anilide: 0.2850 gm. CO<sub>2</sub>, 0.0543 gm. H<sub>2</sub>O  
 0.1000 " " : 0.0112 " N (Kjeldahl).

C<sub>22</sub>H<sub>21</sub>O<sub>3</sub>N<sub>3</sub>. Calculated. C 70.7, H 5.58, N 11.2.

Found. C 70.6, H 5.50, N 11.2.

0.1169 gm. anil: 0.2901 gm. CO<sub>2</sub>, 0.0537 gm. H<sub>2</sub>O.

C<sub>16</sub>H<sub>14</sub>O<sub>3</sub>N<sub>2</sub>. Calculated. C 68.0, H 5.0.

Found. C 67.7, H 5.10.

The hydrolysis of the preceding derivatives with formation of phenylaminomalic acid has not yet been satisfactorily accomplished.

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## STUDIES IN INORGANIC BLOOD PHOSPHATE.

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(Received for publication, July 18, 1921.)

As preliminary to a series of experiments to be noted in the latter part of this paper, a number of inorganic phosphate determinations in the whole blood of rabbits under normal and experimental conditions has been made. These covered first the inorganic phosphate of the blood of normal rabbits fed on the usual laboratory diet of hay, bread, and oats; and second, the curve of inorganic blood phosphate following the intravenous injection of phosphate solutions.

### *Normal Bloods.*

No series of observations on the inorganic phosphate of the blood of normal rabbits has been found in the literature. Iversen's (1) observations on the "acid-soluble" phosphate in normal bloods are prefaced by remarks on his technique in which he states that the investigated bloods (rabbit, guinea pig, rat, cat, etc.) showed 5 to 7 mg. of phosphorus as inorganic phosphate per 100 cc. of blood, whereas in his experimental reports the total "acid-soluble" phosphate is given us about 30 mg. of P per 100 cc. of blood.

The method of Bell and Doisy (2) for the estimation of inorganic blood phosphate was used under the personal direction of Doctor Doisy to whom acknowledgment is gratefully given. The method, a recent one, is simple and as judged by a number of duplicate determinations, accurate. Occasionally a sample of blood was met with in which the color reaction of phosphomolybdic acid failed to appear. Possibly the quantity of ammonium

molybdate added was insufficient to combine with the phosphate in the usual manner. We have often noted a turbidity which is not phosphomolybdate when the ammonium molybdate is added. This undoubtedly indicates another insoluble compound of molybdic acid with the probable failure of the formation of ammonium phosphomolybdate—an essential upon which the determination hinges. It has been found that these anomalous results can be corrected by doubling the quantity of ammonium molybdate added; *i.e.*, 1 cc. of 10 per cent ammonium molybdate in 2 N  $\text{H}_2\text{SO}_4$ .

Oxalated blood was obtained by nicking the marginal ear vein and collecting in amounts of a little over 2 cc. by allowing it to run into a calibrated test-tube containing a few crystals of potassium oxalate. From this tube 2 cc. were pipetted off for estimation. The potassium oxalate was tested for phosphate and was found free from this impurity.

The results of twenty-six readings of normal rabbit blood were as follows:

Mg. per 100 cc. of blood.			
4.1	5.8	4.5	4.4
4.2	6.0	5.3	4.5
4.5	5.1	7.1	3.9
5.4	5.4	6.8	4.5
4.3	5.0	5.7	2.6
5.3	4.6	4.5	4.2
	4.7	4.3	
Average.....4.87 mg. of P as inorganic phosphate per 100 cc. of blood.			

The striking fact in these figures is the uniform level of this substance in the normal blood. All but three of these readings lie between 4 and 6 mg. of P per 100 cc. of blood. The average is seen to be 4.87 mg. The high reading is 7.1 mg. and the low, 2.6 mg. Eleven of these determinations were made in duplicate.

Several of the determinations were carried out on each of three different rabbits over periods up to 50 $\frac{3}{4}$  hours.



Rabbit No.	Date.	Time.	Mg. of P per 100 cc. of blood.
1	Oct. 11	1.30 p. m.	4.1
		2.30 "	4.2
		3.30 "	4.5
	Oct. 12	10.15 a. m.	5.4
		3.00 p. m.	4.3
	Oct. 13	10.15 a. m.	5.3
2	Oct. 12	11.15 a. m.	5.8
		2.00 p. m.	6.0
		3.50 "	5.1
	Oct. 13	10.15 a. m.	5.4
	" 14	2.00 p. m.	5.0
3	Oct. 14	2.00 p. m.	4.6
	Oct. 15	3.30 "	4.7
		3.00 "	4.5

Here again a striking constancy of inorganic phosphate level is shown.

#### *Experimental Injection of Phosphate Solutions.*

The literature of this field of the present study offers a greater number of previous reports, largely because of interest in the phosphorus-calcium balance in the blood and its relation to tetany. The most important work in this connection is that of Binger (3), who plotted a curve of toxicity of injected orthophosphates varying with their pH. He found that in dogs as much as 250 mg. of P per kilo of body weight in the form of orthophosphoric acid could be injected intravenously without development of tetany, whereas 200 mg. in the form of  $\text{Na}_2\text{HPO}_4$  and 150 mg. in the form of  $\text{Na}_3\text{PO}_4$  each caused tetany. He further plotted a curve of serum phosphorus, using Marriott and Haessler's method of estimation, following injection. This curve shows the same prompt fall to normal as the present experiments show. He presents also the inverse curve of calcium that blood analyses in various forms of tetany indicate. Iversen (1) found in the rabbit a similar curve to those about to be reported. His injections were made over a prolonged period. In a later paper (4) he found that both *in vitro* and *in vivo* the red corpuscles take

up gradually a proportion of the acid-soluble phosphate present in the plasma. *In vivo* this means that following the injection of phosphates, the curve of acid-soluble phosphate in the cells falls more slowly than that in the plasma.

All of the present series of experiments were done in the same manner with the exception of the solutions and amounts used. The rabbit, on the usual diet, was weighed and a sample of blood was taken for estimation of inorganic phosphorus as described in the first portion of this paper. The solution at room temperature was then injected as rapidly as possible by gravity from a burette into the marginal vein of the ear not previously employed for the procuring of a blood sample. This injection took about 5 to 8 minutes on the average. Observations for toxic effects were made and blood samples were withdrawn at varying intervals. The solutions used were: (1) M/15  $\text{NaH}_2\text{PO}_4$ ; and (2) a mixture of  $\text{NaH}_2\text{PO}_4 \cdot 4\text{H}_2\text{O}$  and  $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$  so calculated as to present a solution of approximately pH 7.3 and approximately isotonic with the blood.

The curves (Figs. 1 to 8) present the results of the estimations following the injection in 8 of the below 10 experiments.

Experiment No.	Solution 1.	Solution 2.
	cc.	cc.
4	100	
5	75	
6	75	
7	75	
8	75	
9	75	
10	50	
11	50	
19		75
20		75

Experiments 4 and 9 present no curves on account of technical errors in collecting the blood samples. They are included in this report because the former succumbed with positive symptoms of tetany and the latter was the only animal to die without symptoms of tetany.

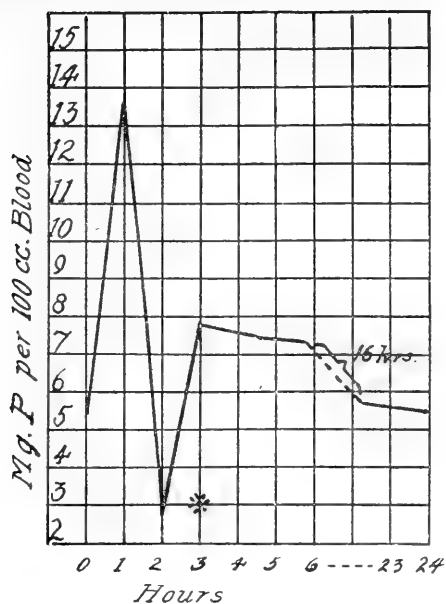


FIG. 1. Experiment 5. 75 mg. of P per kilo— $\text{NaH}_2\text{PO}_4$  solution. No symptoms. The reading marked \* is probably erroneous. The color reaction resembled that mentioned in the text as occasionally disturbing the estimations.

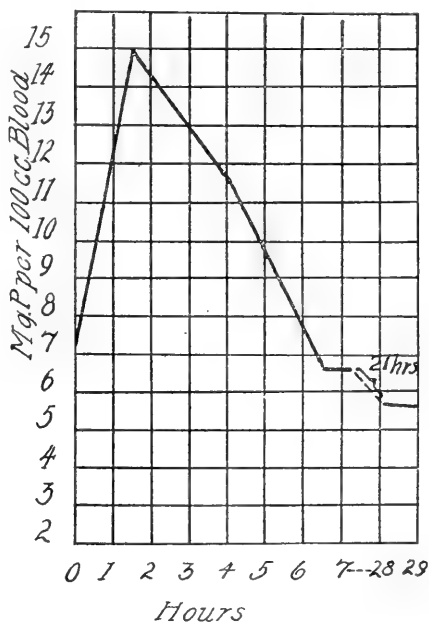


FIG. 2. Experiment 6. 75 mg. of P per kilo— $\text{NaH}_2\text{PO}_4$  solution. Salivation; cyanosis. Survived.

The protocols of Experiments 15 and 18 have been omitted on account of the occurrence of the phenomenon mentioned in the discussion of the Bell and Doisy method. This obviously inval-

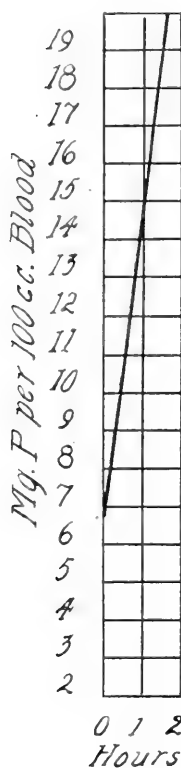


FIG. 3.

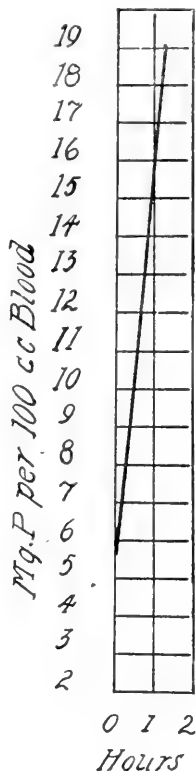


FIG. 4.

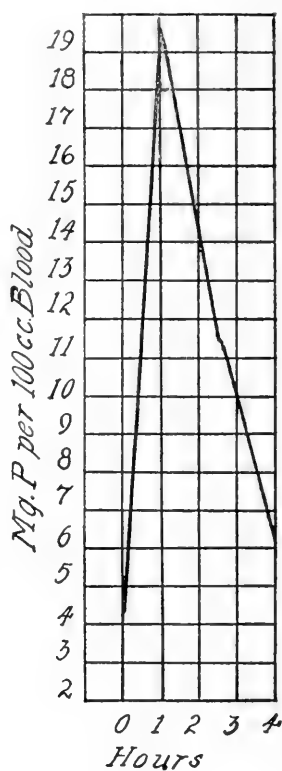


FIG. 5.

FIG. 3. Experiment 7. 75 mg. of P per kilo— $\text{NaH}_2\text{PO}_4$  solution. Tetany; salivation. Dead in about 3 hours.

FIG. 4. Experiment 8. 75 mg. of P per kilo— $\text{NaH}_2\text{PO}_4$  solution. Tetany; salivation. Dead in 18 hours.

FIG. 5. Experiment 10. 50 mg. of P per kilo— $\text{NaH}_2\text{PO}_4$  solution. No symptoms.

idated the results which showed in the former instance a late rise of blood phosphate and in the latter, an experiment on a dog, no rise at all.

There is in these curves a demonstration of the strong tendency of the rabbit's body to maintain the constant level of inorganic blood phosphate found in the estimations on normal rabbits given above. The rapidity, with which concentrations of blood

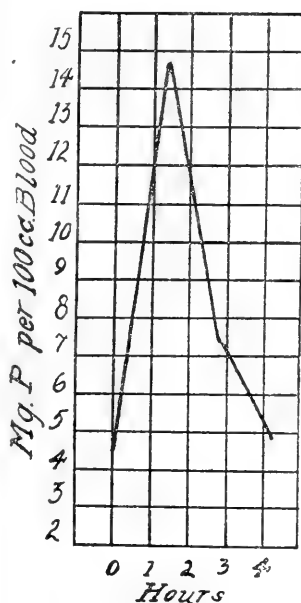


FIG. 6.

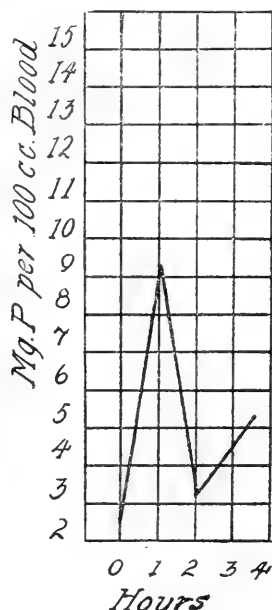


FIG. 7.

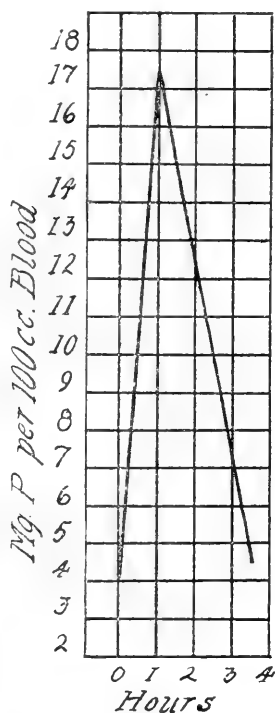


FIG. 8.

FIG. 6. Experiment 11. 50 mg. of P per kilo— $\text{NaH}_2\text{PO}_4$  solution. No symptoms.

FIG. 7. Experiment 19. 75 mg. of P per kilo—mixed phosphate solution. No symptoms.

FIG. 8. Experiment 20. 75 mg. of P per kilo—mixed phosphate solution. Slight tetanic symptoms. Survived.

phosphate four to five times the normal disappear, is extreme. In this connection it must be remembered that the first post injection estimation is made at about 1 hour. Previous to the end of this hour the phosphate concentration must be enormously

higher. Within 4 hours after injection the normal level has been reached. No attempt was made to follow the blood calcium. Somewhat similar experiments on the calcium content of the whole blood of rabbits are reported by Clark (5), with similar results. It is clear that the phosphate-calcium balance is maintained by the organism at a constant level, and that any deviation from this level will be promptly corrected.

The symptoms observed following the injection of massive doses of phosphates deserve mention on account of the interest in the toxicity of these substances. When 100 mg. of P per kilo of  $\text{NaH}_2\text{PO}_4$  were injected (Experiment 4) there was the development of tremors followed by distinct tetanic convulsions and death in 3 hours. These convulsions could be brought on in the rigid extensors of the leg by handling or attempting to flex. Similar observations were made in Experiments 7 and 8 with death in 3 and 18 hours respectively. These two rabbits received 75 mg. of P per kilo of the same substance. Rabbit 9 which also received the last named dosage, died about 12 hours after injection without symptoms of tetany. The only symptoms noted were cyanosis and some form of circulatory collapse which prevented the free flow of blood from the vein. One rabbit (Experiment 20) which received 75 mg. of P per kilo of the mixed phosphate solution showed dyspnea and a questionable spasm of the back and neck muscles. There were no convulsions. An interesting symptom which has for lack of a better name been noted in the curves as "salivation" occurred in several instances. This consisted in a free flow of thin mucoid material from the mouth in the first 2 to 3 hours following injection. Whether or not this represents simply an attempt on the part of the organism to dispose of the excess fluid injected is not clear. No estimation of phosphate in this secretion was made. Nor were controls with the injection of salt solution carried out.

#### *Cod Liver Oil Feeding.*

In connection with recent clinical and experimental reports (6, 7, 8) on the calcification of bone in rickets following the feeding of cod liver oil, it was thought that possibly some light might be thrown on the phenomenon by a similar series of experiments, substituting cod liver oil *per os* for the injection of phosphates. These experiments were entirely negative.

The same general method was employed. Cod liver oil was given by stomach tube in 20 cc. amounts and the inorganic blood phosphate was followed at somewhat longer intervals to allow of absorption. In three such experiments in the rabbit and one in the dog no alteration of inorganic blood phosphate was observed.

*Blood Phosphate and the Calcification of Callus.*

The purpose for which the above experiments were carried out was to form a groundwork on which a surgical problem with biochemical aspects could be carried out. As this work presented negative results it will be but briefly mentioned here.

Provided that the periosteal cell reaction about a fractured bone is normally active, the length of time during which the patient is incapacitated depends primarily on the rate of deposition of calcium salts in the soft callus. With this idea as a basis many experimental and clinical attempts have been made to increase the speed of this calcification.

The present attempt has been based on the following facts. If one adds *in vitro* a solution of phosphates to a solution containing calcium and corresponding in composition to the inorganic composition of the blood, there is precipitated a calcium compound corresponding to the composition of bone (9). Furthermore, the injection of phosphates intravenously results in an immediate fall in the calcium content of the blood, which stays low until the excess phosphate disappears (3). This latter fact suggested the possibility that the reaction definitely known to occur *in vitro* might also occur *in vivo*. The fate of the calcium which disappears from the blood stream is unknown, but it seemed reasonable to assume that it might be directed to a site where there is under ordinary circumstances a tendency for calcium to be deposited. Such a site is, of course, furnished by a soft callus.

The experiments were therefore conducted on the following lines: A bone defect was made with a saw and after varying intervals allowing for the periosteal reaction, the phosphate content of the blood was increased by intravenous injection. The calcification of the fracture was then followed by the x-ray in both the experimental and control animals.

The rabbits were taken in pairs from the same litter and the fore legs x-rayed. A transverse segment of bone about 3 mm. in thickness was then removed from the ulna of each rabbit with the Albee saw, with care to disturb the remaining periosteum as little as possible. Ether anesthesia was used and the wound closed with silk. At the end of an interval varying from 7 to 20 days, the rabbits were again x-rayed. The experimental rabbit then received an injection in the marginal ear vein of a  $\frac{1}{15}$  solution of acid sodium phosphate in the proportion of 50 mg. of phosphorus per kilo of body weight. None of the rabbits showed signs of distress nor was there evidence of tetany. X-rays were taken within the next 6 to 8 hours and again in most instances at later intervals. Eleven pairs of rabbits were subjected to this type of experiment without infection of the wounds. One experiment was conducted by feeding the rabbit cod liver oil instead of injecting phosphate. The results of the experiments showed in no case an undoubted increase of the speed of calcification in the injected rabbits. Two of the above eleven experiments were conducted with the addition of the intraperitoneal injection of a vital stain, sodium alizarin sulfonate (10), as a measure of calcium deposit but in neither of these did this method add anything to the x-ray evidence.

Clark (5), already quoted above, found that an increase of calcium in the blood stream by injection quickly reached the normal level; and that the ingestion of calcium did not affect the normal level in the blood. From these observations one is led to believe that the repetition of the present experiments with an attempt to increase the available calcium would be equally unsuccessful.

#### SUMMARY.

1. The average of twenty-six estimations of the normal inorganic phosphate in the whole blood of rabbits is 4.87 mg. of P per 100 cc.
2. The normal inorganic phosphate of rabbit's blood is practically—that is within biological limits—a constant.
3. An increase by four or five times in the concentration of inorganic phosphate in rabbit's blood returns to normal within 4 hours.



4. The intravenous injection of rabbits with 75 mg. of P per kilo of body weight in the form of  $\text{NaH}_2\text{PO}_4$  will cause tetany in a certain proportion of individuals.

5. On the basis of few experiments, the ingestion of cod liver oil causes no change in the level of inorganic blood phosphate in the rabbit and dog.

6. The intravenous injection in the rabbit of a single massive dose of phosphate has no demonstrable effect on the calcification of callus.

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# CAN "HOME GROWN RATIONS" SUPPLY PROTEINS OF ADEQUATE QUALITY AND QUANTITY FOR HIGH MILK PRODUCTION? III.\*

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(Received for publication, July 25, 1921.)

Supplementary to previous work<sup>1</sup> we have continued our studies on the possibility of furnishing an adequate protein supply to high milk-producing cows from home grown sources. In the work done in 1919-20<sup>1</sup> with alfalfa hay, we maintained by the use of corn starch a like energy supply in the several rations when a change in the grain mixture was made. For example, when ground oats were substituted for ground corn meal the lowered net energy value of the ration was made good by the use of a definite amount of corn starch adjusted according to Armsby's<sup>2</sup> data in which he gives the net energy value of 100 pounds of corn meal as 89.16 therms and of 100 pounds of whole oats as 67.56 therms. When this was done, as our records showed, it was entirely possible to maintain nitrogen equilibrium and high milk production with these liberal milking animals over a period of 16 weeks. The possible effect of a lowered energy intake through the substitution of ground whole oats for ground corn meal in the ration with maintenance of a constant protein level but with no starch additions was now studied.

\* Published with the permission of the Director of the Wisconsin Agricultural Experiment Station.

<sup>1</sup> Hart, E. B., and Humphrey, G. C., *J. Biol. Chem.*, 1919, xxxviii, 515; 1920, xlv, 189.

<sup>2</sup> Putney, F. S., and Armsby, H. P., *Pennsylvania State College Bull.* 143, 1916.

Such an experiment would touch practice more closely than our earlier experiments did, as the common farm procedure would be to use either a single grain such as whole oats or a mixture of grains, but without the addition of starch.

#### EXPERIMENTAL.

In these experiments we worked with the corn, barley, and oat grains, used singly and supplemented with corn silage and alfalfa hay. The alfalfa was grown in southern Wisconsin and was taken from the second cutting.

Since the protein content of barley was intermediate to that of the corn and oat grains used, we chose the barley ration as the standard to which the other rations must conform with respect to protein. For example, 50 pounds of the barley grain ration consisted of 10 pounds of grain, 10 pounds of alfalfa, and 30 pounds of corn silage. The corn grain mixture consisted of 10.6 pounds of corn meal, 10 pounds of alfalfa, and 30 pounds of silage and contained the same amount of total protein as the barley ration. The oat grain ration consisted of 9.1 pounds of whole oats, 10 pounds of alfalfa, and 30 pounds of silage and furnished the same amount of protein ( $N \times 6.25$ ) as the other rations. The 50 pounds of barley ration furnished 16.37 therms; the 50.6 pounds of corn ration furnished 17.24 therms; while the 49.1 pounds of oat grain ration furnished but 14.43 therms.

Much to our surprise these differences in the net energy values of the rations, particularly the oat ration as compared with the others, were sufficient to determine whether these high milk-producing cows would be in negative or positive nitrogen balance. The corn and barley rations were ample both in protein and net energy content for high milk production over the periods of observation; but the oat ration was not generous enough in its net energy content for these lactating animals—the effect being manifested by distinct negative nitrogen balances during the period of 4 weeks observation. Evidently protein was being destroyed as a source of energy during the feeding of this lower therm-containing ration.

Cow 3 weighed approximately 1,000 pounds and was producing daily 40 pounds of milk containing 3 per cent of fat. According to Armsby's standard such a cow would require for maintenance

and the production of this amount of milk a daily intake of 14.4 therms. Actually she was receiving 14.43 therms per day, but with that amount of energy, nitrogen equilibrium was not maintained; for while Armsby's standard requires 2.22 pounds daily of digestible *true* protein for a cow with the producing capacity of No. 3, she was receiving but 1.68 pounds of digestible *true* protein. The two other animals involved in this inquiry showed similar negative nitrogen balances on the whole oat ration but positive balances or equilibrium on the barley and corn rations, in which the protein content was the same as in the oat ration but the net energy supply 2 to 3 therms higher per day.

Animal 1 was a pure bred Holstein, No. 2 a grade Jersey, and No. 3 a grade Guernsey. They weighed respectively as follows: No. 1, initial weight 1,502 pounds, final weight 1,465 pounds; No. 2, initial weight 997 pounds, final weight 1,034 pounds; No. 3, initial weight 1,038 pounds, final weight 995 pounds. The methods of analysis and quantitative collection of excreta have been described in earlier publications. Each ration was fed for a period of 4 weeks with a preliminary feeding period of 5 days before quantitative collection of excreta was begun. There was no preliminary feeding period when the rations were changed. Each cow was allowed what she would completely consume of the mixed ration.

The ration used contained the following percentages of nitrogen:

	Nitrogen. <i>per cent</i>
Corn grain.....	1.57
Oat grain.....	1.82
Barley grain.....	1.66
Corn silage.....	0.36
Alfalfa hay.....	2.65

The data on nitrogen balances are presented in Tables I to III inclusive.

From the data presented in the tables it is clearly evident that there was not only a distinct negative nitrogen balance during the oat grain period of feeding, when the therm intake was reduced, but that there was a marked increased destruction of protein in this period as shown in the greatly increased urinary nitrogen output exhibited by each animal. No. 1 increased her urinary

nitrogen output 50 per cent above the urinary nitrogen output on the corn grain ration and increases of nearly similar magnitude were shown by Nos. 2 and 3.

These data are not to be interpreted as indicating an inferiority of the oat proteins as compared with those of the corn or barley grain since all available evidence<sup>1</sup> points towards an approximately equal supplementary efficiency for the cereal grain proteins.

TABLE I.

*Record of Nitrogen Balance, Milk Production, Etc., in Animal I.*

Date.	Nitrogen.					
	Intake.	Feces.	Urine.	Milk.	Balance.	Milk per week.
Barley grain ration.						
	gm.	gm.	gm.	gm.	gm.	lbs.
Dec. 14-20.....	1,712.9	647.7	570.9	585.8	-91.5	248
" 21-27.....	1,712.9	715.7	540.2	547.2	-90.2	236
" 28-Jan. 3.....	1,712.9	672.8	458.2	550.3	+31.6	241
Jan. 4-10.....	1,850.1	698.3	423.1	559.0	+169.7	244
Corn grain ration.						
Jan. 11-17.....	1,850.1	728.3	472.9	569.0	+79.9	252
" 18-24.....	1,850.1	788.9	436.8	569.6	+54.8	257
" 25-31.....	1,850.1	702.5	484.5	586.1	+77.0	258
Feb. 1-7.....	1,850.1	702.5	525.6	571.7	+50.3	252
Oat grain ration.						
Feb. 8-14.....	1,850.1	583.4	668.9	555.9	+41.9	244
" 15-21.....	1,850.1	616.5	707.0	556.9	-30.3	244
" 22-28.....	1,850.1	622.9	793.0	539.7	-105.5	236
Mar. 1-7.....	1,850.1	651.8	817.3	540.1	-159.1	232

Another important point coming from the collection of such data as here presented are differences in urinary nitrogen elimination which are often observed with approximately similar absorption from the intestine. Apparently in individuals different rates of deamination and destruction of important carbon nuclei are taking place which may be closely related to the often observed differences in the efficiency of a protein mixture with different animals. For example, in these trials No. 2 during the corn

ration period was absorbing approximately 1,000 gm. of nitrogen per week and eliminating 480 gm. in the urine, while Animal 3 during the same period was absorbing practically the same amount of nitrogen but eliminating only 410 gm. per week; presumably the energy requirement was amply covered in both individuals. It is in this direction; namely, the rates of intermediary nitrogen metabolism and the special tissues involved in producing these different rates, that we must look for an

TABLE II.  
*Record of Nitrogen Balance, Milk Production, Etc., in Animal II.*

Date.	Nitrogen.					
	Intake.	Feces.	Urine.	Milk.	Balance.	Milk per week.
Barley grain ration.						
	gm.	gm.	gm.	gm.	gm.	lbs.
Dec. 14-20.....	1,575.7	549.6	409.2	491.3	+125.6	185
" 21-27.....	1,575.7	588.1	524.3	444.5	+18.8	167
" 28-Jan. 3.....	1,575.7	602.0	449.0	440.2	+84.5	188
Jan. 4-10.....	1,575.7	603.0	450.7	445.0	+77.0	194
Corn grain ration.						
Jan. 11-17.....	1,575.7	560.1	444.8	439.1	+131.7	180
" 18-24.....	1,575.7	620.9	492.8	434.9	+27.1	160
" 25-31.....	1,575.7	567.4	497.8	439.8	+70.7	185
Feb. 1-7.....	1,575.7	563.6	476.6	437.3	+98.2	180
Oat grain ration.						
Feb. 8-14.....	1,575.7	503.6	628.9	433.3	+9.9	177
" 15-21.....	1,575.7	488.9	614.1	431.1	+41.6	173
" 22-28.....	1,575.7	494.0	663.6	428.5	-10.4	174
Mar. 1-7.....	1,575.7	446.6	713.5	439.3	-23.7	175

explanation of differences in the efficiency of individuals in respect to protein utilization. While protein constitution is of primary importance in this respect, the additional factor of the individual rate of intermediary metabolism will come into play especially in the application to practice of a mathematical standard. This factor would assume special importance where the protein allowance was not liberal and was reduced to a standard formulated from data obtained with the "best" individuals.

TABLE III.  
*Record of Nitrogen Balance, Milk Production, Etc., in Animal III.*

Date.	Nitrogen.					
	Intake.	Feces.	Urine.	Milk.	Balance.	Milk per week.
Barley grain ration.						
	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>lbs.</i>
Dec. 14-20.....	1,712.9	664.2	415.5	638.7	-5.3	316
" 21-27.....	1,712.9	661.2	387.8	612.8	+49.1	308
" 28-Jan. 3.....	1,712.9	689.1	318.6	639.7	+65.5	341
Jan. 4-10.....	1,712.9	638.1	392.5	640.9	+41.4	334
Corn grain ration.						
Jan. 11-17.....	1,712.9	731.8	366.7	628.1	-13.7	317
" 18-24.....	1,712.9	746.3	437.8	588.3	-59.5	321
" 25-31.....	1,712.9	682.0	447.4	570.3	+13.2	319
Feb. 1-7.....	1,719.9	698.4	397.0	565.5	+52.0	308
Oat grain ration.						
Feb. 8-14.....	1,712.9	580.8	558.6	572.1	+1.4	302
" 15-21.....	1,712.9	590.0	564.3	562.1	-3.5	291
" 22-28.....	1,712.9	570.2	647.0	553.9	-58.2	278
Mar. 1-7.....	1,712.9	549.7	642.6	555.4	-34.8	279

TABLE IV.  
*Composition of Milk and Average Daily Flow in a Selected Week of Each Period.*

	Dec. 28	Jan. 25	Feb. 22
Animal I.			
Total solids, <i>per cent.</i> .....	12.98	12.03	11.85
Fat, <i>per cent.</i> .....	3.60	3.40	3.55
Nitrogen, <i>per cent.</i> .....	0.51	0.50	0.49
Milk daily, <i>lbs.</i> .....	35.00	35.00	34.00
Animal II.			
Total solids, <i>per cent.</i> .....	14.38	13.92	13.39
Fat, <i>per cent.</i> .....	4.80	4.70	4.70
Nitrogen, <i>per cent.</i> .....	0.52	0.53	0.51
Milk daily, <i>lbs.</i> .....	27.00	26.00	25.00
Animal III.			
Total solids, <i>per cent.</i> .....	11.75	10.79	10.84
Fat, <i>per cent.</i> .....	3.00	2.80	3.00
Nitrogen, <i>per cent.</i> .....	0.42	0.38	0.39
Milk daily, <i>lbs.</i> .....	48.00	45.00	40.00



In Table IV are recorded the composition of the milk and the average daily flow in a selected week of each period. The milk composition as well as flow was well maintained for the period of observation, but there can be no doubt but that a long continued feeding period on the oat ration, involving inadequate energy intake, would ultimately have affected milk secretion and milk composition.

#### SUMMARY.

1. Data are presented which show that it is entirely possible when feeding equal but limited amounts of protein to maintain nitrogen equilibrium and high milk production in dairy cows with a ration composed of either barley or corn supplemented with corn silage and alfalfa hay, but not with the whole oat grain so supplemented.

2. Previous records had indicated this possibility with all cereal grains, but only when the deficient net energy content of the oat grain ration was made good by the use of corn starch.

3. The oat grain ration contained 14.43 therms per 49.1 pounds; the corn ration contained 17.24 therms per 50.6 pounds; both rations contained exactly the same amount of protein; yet this difference in the energy supply of the two rations was sufficient to produce a positive nitrogen balance on the 17.24 therms but a negative nitrogen balance on the 14.43 therms.

4. In practice, a mixture of the corn grain or the barley grain with the oat grain, 50 per cent of each, would very probably make up this deficiency in net energy.



## STUDIES ON BLOOD SUGAR.

### THE TOTAL AMOUNT OF CIRCULATING SUGAR IN THE BLOOD IN DIABETES MELLITUS AND OTHER CONDITIONS.\*

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(Received for publication, July 18, 1921.)

In this paper observations are reported on the total amount of sugar in the circulating blood, and on its relative distribution between plasma and corpuscles in a series of normal persons, of diabetic patients, and of patients with other diseases.

Numerous experimenters have studied the distribution of sugar per unit volume of plasma and corpuscles. The subject was carefully reviewed by Gradwohl and Blaivas, in 1917, and more recently by Wishart. According to the statements of certain previous workers, the corpuscles sometimes contain little or no sugar; according to others, the sugar content of the corpuscles does not differ greatly from that of the plasma; another belief has been that the corpuscles take up sugar more slowly and retain it longer than does the plasma so that the corpuscular sugar is low in the early stages of hyperglycemia, but above that of the plasma in the declining stages. The preponderance of experience is that the sugar content per unit volume of corpuscles is usually a little below that of the plasma. According to Wishart the discrepancy in favor of the plasma generally becomes greater as the blood sugar rises.

The desirability of knowing the total amount of circulating sugar and its distribution between the total volume of plasma

\* This paper is Number 19 of a series of studies, on the physiology and pathology of the blood, from the Harvard Medical School and allied hospitals, a part of the expense of which has been defrayed from a grant from the Proctor Fund of the Harvard Medical School for the study of chronic diseases.

and corpuscles was suggested by Epstein and Baehr in this country in 1914. These observers produced rapid changes in the blood volume of experimental animals. By diluting the blood the percentage concentration of sugar diminished, although the total amount of circulating sugar remained constant or was increased. They concluded, therefore, that in diabetes the total amount of circulating sugar must bear a more definite relation to glycosuria than the mere concentration of sugar for each unit volume of blood. Sansum and Woodyatt, in 1917, also determined the importance of the total blood sugar in establishing and regulating the rate of glycosuria and that the concentration of sugar in the plasma was of but little importance in this respect. No data have heretofore been published, however, which record the normal amount of circulating sugar in man, nor the variations from the normal that may occur in diabetes and other conditions. We obtained data on the subject by the following method.

The total volume of the circulating blood and the total corpuscular and plasma volume were determined by the vital red method of Keith, Rowntree, and Geraghty. One of us (Bock) has recently published a discussion of the advantages and disadvantages of this method. The sugar concentration of oxalated whole blood and oxalated unhemolyzed plasma for each 100 cc. was obtained at the same time by the method of Folin and Wu. Knowing the amount of sugar to 100 cc. of blood and plasma, and the total volume of circulating blood and plasma, the total amount of sugar in the whole blood and plasma was readily obtained. The differences between the total amount of sugar and that found in the plasma represented the total corpuscular sugar. The results of our observations are incorporated in Tables I to IV.

In Table I is recorded the total amount of sugar in the blood of seven normal persons; the amount varied between 7.54 and 2.50 gm. The plasma sugar varied between 4.85 and 1.64 gm. The corpuscular sugar varied between 3.85 and 0.69 gm. The average total amount of blood sugar for the group was 5.18 gm. The average plasma sugar was 3.29 gm. The average corpuscular sugar was 1.89 gm. The marked variations encountered in the individual cases probably depended, among other reasons, on wide differences in the area of body surface, and in weight, and on the fact that the estimations were made with

disregard for the time or nature of the previous meal. However, it seems logical to conclude from these data that by the methods employed the normal total amount of blood sugar does not exceed 7.50 gm., and that the normal plasma sugar is usually considerably greater than the corpuscular sugar, but does not exceed 4.85 gm.

In Table II are recorded similar observations in a group of nine diabetic patients. As was to be expected, in diabetics, as well as in normal persons, there were fluctuations in the amount of circulating sugar. The highest amount of sugar in the blood was 15 gm., the lowest 6.81 gm. The plasma, relatively

TABLE I.  
*Normal Persons.*

Case.	Sex.	Weight. <i>kg.</i>	Blood volume. <i>cc.</i>	Plasma volume. <i>cc.</i>	Corpuscular volume. <i>cc.</i>	Blood sugar mg. per 100 cc.		Total blood sugar. <i>gm.</i>	Total plasma sugar. <i>gm.</i>	Total corpuscu- lar sugar. <i>gm.</i>
						Blood.	Plasma.			
1	M.	82	6,218	3,731	2,487	120	130	7.54	4.85	2.69
2	M.	63	5,758	3,628	2,130	130	100	7.48	3.63	3.85
3	F.	55	5,750	3,970	1,780	100	90	5.75	3.55	2.20
4	F.	60	4,500	3,200	1,300	100	100	4.50	3.20	1.30
5	F.	60	4,460	3,120	1,330	90	100	3.81	3.12	0.69
6	F.	60	4,230	2,750	1,480	110	110	4.65	3.02	1.63
7	F.	60	3,570	2,340	1,230	70	70	2.50	1.64	0.86
Average.....								5.18	3.29	1.89

contained much more sugar than did the corpuscles. Thus the highest plasma sugar content was 10.78 gm., and the lowest 4.75 gm., while the highest corpuscular sugar content was 4.22 gm., and the lowest was 1.09 gm. The average total amount of blood sugar for the group was 8.95 gm. The average total plasma sugar was 6.72 gm. The average corpuscular sugar content was 2.23 gm.

These findings are evidence that the blood, especially the plasma in diabetes, is a vehicle for the transportation of sugar from the body cells which are unable to burn or store it, to the kidney which excretes it. The blood corpuscles, as a whole, are but

little concerned with such transportation of sugar and do not contain an increase in sugar proportional to that found in the plasma. Whether this depends largely, as Wishart has suggested, on the fact that glucose is more freely soluble in the plasma than in the corpuscular substance, or on other considerations, is uncertain. In any event, it seems logical to conclude with Wishart that analyses of plasma of the total amount of sugar and of its concentration for each 100 cc. are preferable to those of whole blood.

TABLE II.  
*Patients with Diabetes.*

Case.	Sex.	Weight. kg.	Blood volume. cc.	Plasma volume. cc.	Corpuscular volume. cc.	Blood sugar mg. per 100 cc.		Total blood sugar.	Total plasma sugar	Total corpuscular sugar.
						Blood.	Plasma.			
1	F.	70.0	4,690	2,990	1,700	320	360	15.00	10.78	4.22
2	M.	47.0	4,030	2,500	1,530	170	190	6.86	4.75	2.11
3	M.	43.0	3,880	2,440	1,440	190	220	7.37	5.36	2.01
4	M.	41.5	3,784	2,365	1,419	180	220	6.81	5.20	1.61
5	M.	62.0	3,760	2,480	1,280	220	240	8.24	5.95	2.29
6	M.	54.0	3,590	2,370	1,220	200	250	7.18	5.92	1.26
7	M.	54.5	3,363	2,200	1,163	340	380	11.43	8.36	3.07
8	M.	50.0	3,340	2,140	1,200	240	260	8.02	5.56	2.46
9	F.	35.5	2,930	2,200	730	330	390	9.67	8.58	1.09
Average.....								8.95	6.72	2.23

The findings shown in Table II demonstrate, moreover, that blood sugar concentration expressed as mg. per 100 cc. of blood or plasma may give misleading information with regard to the total amount of circulating sugar, as Epstein and Baehr and Sansum and Woodyatt have suggested. For example, in Cases 1, 7, and 9, respectively, the sugar concentrations were 360, 380, and 390 mg. of sugar per 100 cc. of plasma. In Case 1 the plasma sugar concentration was the lowest of the three, with 10.8 gm. of sugar in the total plasma. In Case 7 the sugar in the total plasma was 8.36 gm., and in Case 9, in which the sugar concentration was the highest of the series, the sugar in the total plasma was only 8.6 gm.

The relation between the total amount of plasma sugar and the total amount of urine sugar was studied in a few cases. For this purpose the urine was collected for the 24 hour period in the middle of which the blood volume and sugar determinations were made. The sugar excretion was titrated by the Benedict method on the urine in cases which yielded a positive reaction to Benedict's qualitative test. The findings are recorded in Table III.

The threshold at which glucose appeared in the urine of patients with diabetes seemed to lie between 5.2 and 5.36 gm. of total plasma sugar. One diabetic patient whose plasma sugar concentration was 190 gm. per 100 cc. had a low plasma volume

TABLE III.

*The Relation between Total Plasma Sugar and Sugar Excretion.*

Case.	Diagnosis.	Plasma sugar mg. per 100 cc.	Total plasma sugar.	Body weight. "W"	Urine sugar per liter. "C"	Urine sugar per 24 hours. "D"	Urine $\frac{D}{W} \sqrt{C}$
			gm.	kg.	gm.	gm.	
1	Normal.	130	4.85	82.0	0	.0	
2	Diabetes.	190	4.75	47.0	0	0	
3	"	220	5.20	41.5	0	0	
4	"	220	5.36	43.0	Trace.	Trace.	
5	"	250	5.92	54.0	5.00	27.50	1.14
6	"	390	8.58	35.5	13.90	21.54	2.26
7	"	360	10.78	70.0	29.40	52.92	4.10

and thus a low total amount of sugar. This probably explains why the patient did not excrete sugar in the urine with so high a glycemia.

Three patients excreted titratable amounts of sugar. The total sugar excretion did not have any obvious connection with either the concentration of sugar in the plasma or with the total amount. The sugar excretion was therefore estimated according to the formula which Ambard has used in studying urea and chloride excretions and which Fitz and Van Slyke used as the basis of their acid excretion formula. Sugar excretion recorded in this manner was not proportional to the plasma sugar concentration. The total plasma sugar, however, appeared to be related to sugar excretion expressed in this form (Chart 1).

These observations are too few to be very conclusive. They suggest, however, that the total amount of plasma sugar offers a more rational basis of comparison with sugar excretion than does the plasma sugar concentration alone, and that it may be possible to work out a formula which will express mathematically the relationship between the sugar circulating in the blood and that excreted in the urine.

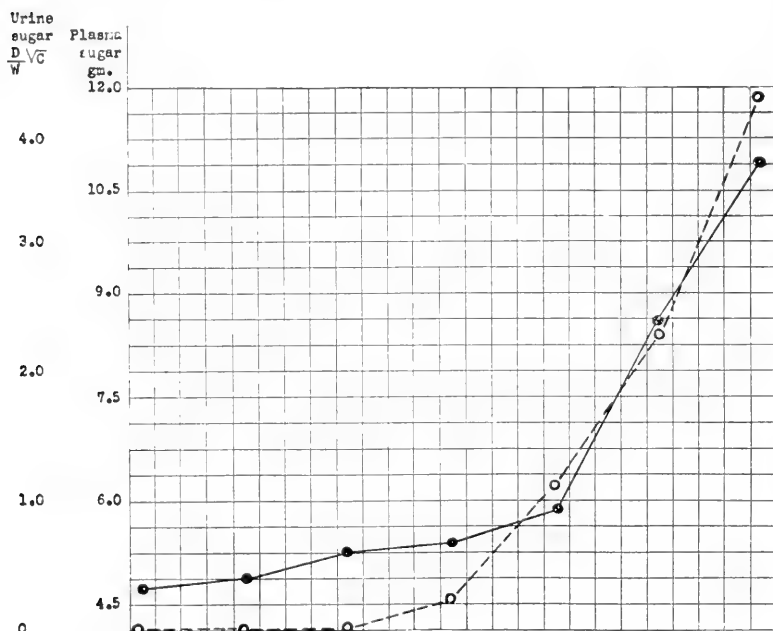


CHART 1. The relationship between total plasma sugar and the excretion of sugar in the urine.

The most striking feature of the miscellaneous cases tabulated in Table IV is the great difference in the estimation of total blood sugar. Thus a patient with polycythemia had 16.20 gm. of sugar in the blood, while a patient with nephritis had only 2.06 gm. of sugar in the blood. The average for this group of patients with miscellaneous diseases as a whole is lower than the average for normal persons (Table I), probably on account of the number of anemic patients who were studied.



It is of especial interest that the patient with polycythemia had more sugar circulating in the blood and more in the corpuscles than any of the diabetic patients, although the urine was normal. The total plasma sugar, on the other hand, was within normal limits. This suggests that sugar contained in the corpuscles is tightly bound to them in some manner and has little effect on the production of glycosuria.

In all three groups of cases the corpuscular sugar content was parallel with the corpuscular volume. Apparently, therefore,

TABLE IV.  
*Patients with Miscellaneous Diseases.*

Case.	Sex.	Weight. <i>kg.</i>	Diagnosis.	Blood volume. <i>cc.</i>	Plasma volume. <i>cc.</i>	Corpuscular vol- ume. <i>cc.</i>	Blood sugar mg. per 100 cc.		Total blood sugar. <i>gm.</i>	Total plasma sugar. <i>gm.</i>	Total corpuscular sugar. <i>gm.</i>
							Blood.	Plasma.			
1	M.	60.5	Polycythemia.	8,540	3,240	5,300	190	150	16.20	4.85	11.35
2		45.3	Leukemia.	5,060	3,460	1,590	90	80	4.55	2.77	1.78
3	F.	60.5	Secondary anemia.	4,470	3,400	1,070	110	110	4.92	3.76	1.16
4	M.	65.0	Nephritis.	4,390	3,170	1,270	110	80	4.83	2.54	2.29
5		50.0	Leukemia.	4,030	3,140	890	100	100	4.03	3.14	0.89
6	F.	43.5	Pernicious anemia.	3,070	2,730	340	120	120	3.68	3.28	0.40
7	F.	43.5	" "	3,050	2,600	460	120	120	3.66	2.60	1.06
8	F.	54.5	" "	2,740	2,330	410	120	120	3.29	2.80	0.49
9	M.	54.5	Nephritis.	2,680	2,140	530	80	90	2.14	1.92	0.22
10	M.	45.0	"	2,580	2,200	380	80	80	2.06	1.76	0.30
11	F.		Pernicious anemia.	2,460	2,180	280	100	90	2.46	1.96	0.50
Average.....									4.71	2.85	1.86

all blood corpuscles contain a certain amount of sugar which is fixed within rough limits. If the number of corpuscles is greatly increased, the total corpuscular sugar is also increased; the corpuscular content of sugar is low when the number of corpuscles is diminished; when sugar is added to the circulating blood it is found largely in the plasma and to a much less extent in the corpuscles. Glycosuria does not occur unless the total plasma sugar is above a certain limit, regardless of what the sugar content of the corpuscles or whole blood may be.

## SUMMARY.

The total amount of sugar in the blood of seven normal persons varied but did not exceed 7.5 gm. The plasma sugar was almost always considerably greater than the corpuscular sugar, but it did not exceed 4.85 gm. The total amount of sugar in the blood of nine diabetic patients also varied considerably. The highest blood sugar content estimated was 15 gm., and the highest plasma sugar was 10.78 gm.

The plasma of the diabetic bloods, relatively, contained much more sugar than did the corpuscles. This suggests that the plasma in diabetes is a vehicle for the transportation of sugar from the body cells, which are unable to burn or store it, to the kidney which excretes it, and that the blood corpuscles are but little concerned with such transportation of sugar, a statement which is supported by the fact that the sugar content of the individual corpuscle tends to be fixed within rough limits. If the number of corpuscles is increased, as in polycythemia, the total corpuscular sugar is increased. If the number of corpuscles is much diminished, as in anemia, the amount of corpuscular sugar is diminished. Glycosuria does not occur unless the plasma sugar exceeds a certain threshold.

Blood sugar concentration expressed as mg. per 100 cc. of blood or plasma may give misleading information with regard to the total amount of circulating sugar. The threshold at which glucose appeared in the urine of the diabetic patients of this series seemed to lie between 5.20 and 5.36 gm. of total plasma sugar. The total plasma sugar offered a more rational basis of comparison with sugar excretion than did the plasma sugar concentration alone.

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## EFFECT OF HEATING THE ANTISCORBUTIC VITAMINE IN THE PRESENCE OF INVERTASE.

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(Received for publication, July 13, 1921.)

A number of papers which have appeared recently have suggested that the presence of enzymes in food may influence the stability of the antiscorbutic enzyme. Givens and McClugage (1) state:

"The influence of heat upon the antiscorbutic vitamine appears to be related not only to the degree of temperature but to the duration of the treatment, the reaction, the enzymes present, and the manner of heating."

They base their conclusion as to this enzyme action partly upon experiments showing that potatoes baked for a short time at high temperature and then dried at 35-40°C. retain a greater amount of the antiscorbutic vitamine than those dried at 35-40°C. without previous heating.

In this same article, Givens and McClugage refer especially to oxidases present in the potato. Ellis, Steenbock, and Hart (2) have shown that drying cabbage in an atmosphere of CO<sub>2</sub> for 35 hours at 65°C. does not prevent the destruction of the antiscorbutic vitamine. Oxidizing agents such as hydrogen peroxide and potassium permanganate cause its destruction.

Anderson, Dutcher, Eckles, and Wilbur (3) state that oxidation is a more important factor than heat in the destruction of the antiscorbutic vitamine. Bubbling air through cow's milk at 145°F. for 30 minutes causes some destruction, but the destruction is more marked when oxygen or hydrogen peroxide is used.

These experiments, together with the fact, as shown by Harden and Robinson (4) and Givens and Macy (5), that dehydrated orange juice retains its antiscorbutic value after 2 years, whereas untreated orange juice deteriorates appreciably within 3 months (6) indicate strongly that enzyme action may be an important factor in the gradual destruction of the antiscorbutic vitamine.

## EXPERIMENTAL.

In the present study the authors have heated orange juice, both in the presence and absence of an enzyme. The temperatures chosen were 38°, 55°, and 76°C. If enzyme activity causes destruction of the vitamine, presumably disappearance of the latter would be most rapid at 55°C., the temperature at which the activity of the enzyme is greatest.

The enzyme selected was invertase, since it is present in the natural orange juice. The experiments conducted show conclusively that this enzyme does not decrease the value of the antiscorbutic vitamine. This does not preclude the possibility that other enzymes may have such an effect, and it is hoped that experiments along this line may be continued with an oxidizing enzyme.

The antiscorbutic vitamine was separated from orange juice<sup>1</sup> by the method of Hess and Unger (6) with 96 per cent alcohol which precipitated the enzyme and thus separated it from the vitamine. The extract was tested on a solution of sucrose and gave a negative test for invertase.

The invertase was prepared by a method described by Hudson and Paine (7). It was dried on filter paper and 25 mg. were added to each 100 cc. of orange extract.

4 cc. of orange extract, prepared fresh about every 10 days, were fed to each guinea pig daily for the first 46 days of the experiment. As none of the guinea pigs receiving the extract showed any symptoms of scurvy at that time, the amount fed was reduced to 3 cc. for the following 18 days. As there were still no indications of scurvy in any of the animals, and as the experiment had to be terminated within another month, the amount fed daily was reduced to 1.5 cc. per animal.

Table I gives the amount fed for the various periods, and the time each portion was heated.

The basal diet used, consisted of equal parts by weight of alfalfa meal and wheat flour, with powdered milk which had previously been heated to 95°C. for 1 hour. These three were mixed thoroughly together and made into a soft paste with water.

<sup>1</sup> The oranges in these experiments were generously supplied by the Research Laboratory of the California Fruit Growers Exchange, Corona, California.

The allowance of milk was about 3 cc. per day per animal. Cracked oats were kept constantly before the animals.

Table II gives the diets fed the various guinea pigs.

The animals were weighed every morning before feeding. In Table III are recorded the weights for every fifth day.

TABLE I.  
*Treatment of Orange Extract.*

Time heated.	Amount fed.	Period fed.
<i>hrs.</i>	<i>cc.</i>	<i>days</i>
4	4	11
2	4	21
4	4	15
4	3	18
4	1.5	25

TABLE II.  
*Diets.*

No. of animal.	Basal diet.	Invertase.	Orange extract.	Temperature heated.
				$^{\circ}\text{C}.$
29	+	—	—	
25	+	+	—	
27	+	—	+	
33 and 34	+	—	+	38
31 and 37	+	+	+	38
28 and 35	+	—	+	55
32 and 36	+	+	+	55
30 and 38	+	—	+	76
26 and 39	+	+	+	76

#### SUMMARY.

Invertase does not contribute to the destruction of the antiscorbutic vitamine when heated with the vitamine for 4 hours at  $76^{\circ}$ ,  $55^{\circ}$ , or  $38^{\circ}\text{C}.$

Heating for 4 hours at a temperature of  $76^{\circ}\text{C}.$  either in the presence of invertase or in its absence, causes a more rapid destruction of the vitamine than heating at  $55^{\circ}\text{C}.$  Heating for 4 hours at  $38^{\circ}\text{C}.$ , does not cause an appreciably greater loss of antiscorbutic value than keeping at room temperature.

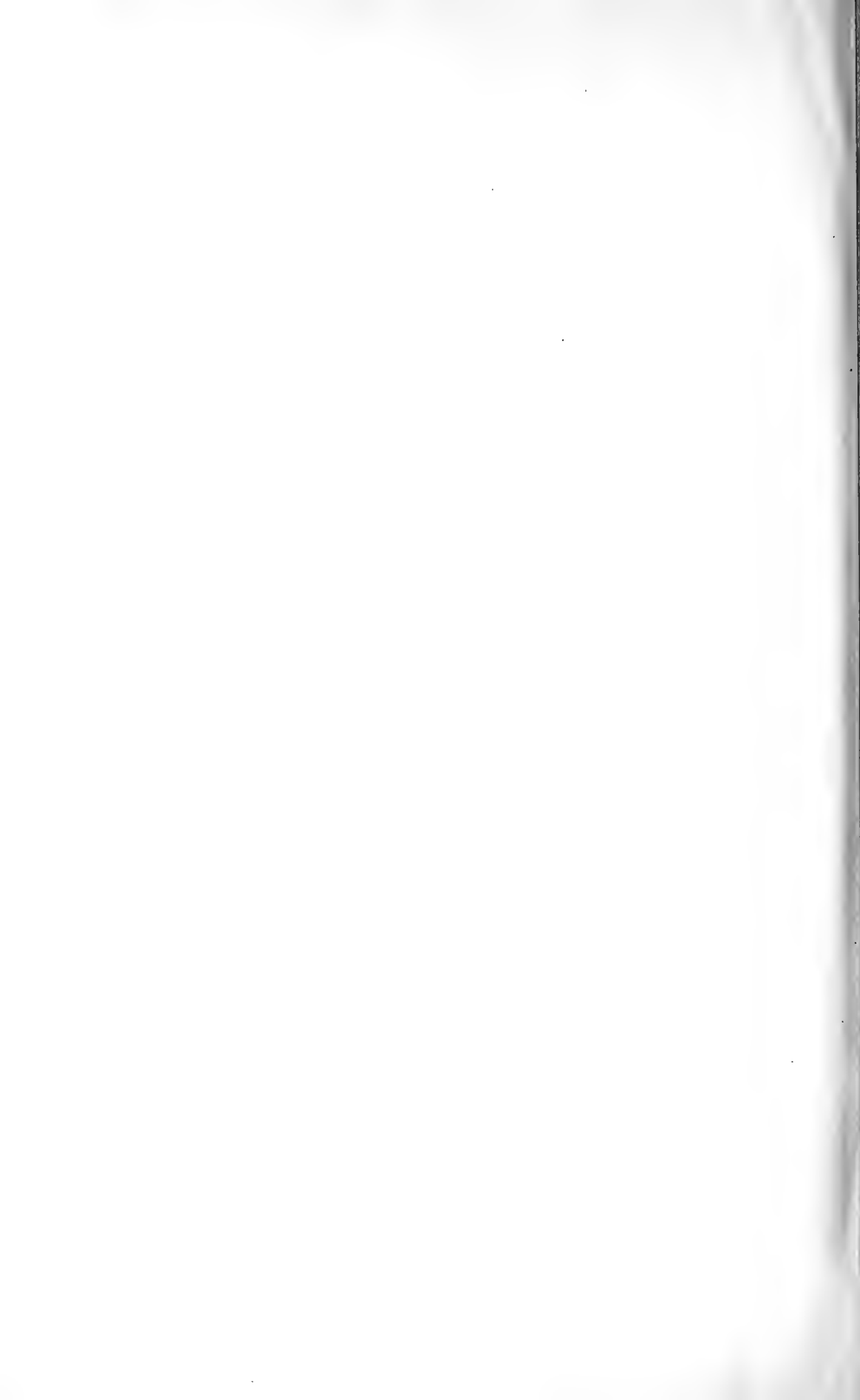
Date.	No. 29	No. 25	No. 27	No. 33	No. 34	No. 31	No. 37	No. 28	No. 35	No. 32	No. 36	No. 30	No. 38	No. 26	No. 39
Mar. 19	374	536	385	463	304	514	252	378	348	454	305	344	376	357	387
" 23	383	534	401	508	329	555	227	397	355	479	325	353	386	358	386
" 28	395	554	427	502	350	584	242	417	379	487	335	370	420	377	409
Apr. 2	414	562	438	538	386	607	244	432	396	500	350	394	433	379	424
" 7	425	548	460	575	403	640	250	449	410	521	374	422	463	393	447
" 12	379	462	486	567	420	654	255	457	419	538	374	447	487	409	459
" 17	364	371	504	597	417	655	274	484	436	551	389	456	517	407	477
" 19	335	335													
" 22	Dead.	Dead.	485	625	422	659	283	510	450	564	394	475	532	425	496
" 27			508	633	450	662	271	507	455	578	409	477	563	417	518
May 2			512	658	455	680	255	533	473	587	429	475	537	437	530
" 7			494	687	469	669	262	535	472	604	446	475	547	437	541
" 12			506	680	478	681	270	522	480	602	457	507	532	445	560
" 17			519	702	485	707	285	511	490	614	469	528	537	463	570
" 22			528	707	489	700	290	544	496	630	480	538	531	456	566
" 27			543	713	504	686	295	544	497	632	490	535	502	464	571
June 1			572	720	525	695	307	565	518	647	518	441	463	452	568
" 6			557	717	502	709	297	545	517	642	524	376	377	430	521
" 10											329	335	Dead.	343	438
" 11			550	704	501	705	298	510	494	541	518	Dead.	Dead.	272	361
" 16			510	646	482	702	280	425	445	445	452			Dying.	Dying.



In all cases except Guinea pig 32 which was fed on orange extract plus invertase heated at 55°C., the animals receiving orange extract heated in the presence of the enzyme were in a less advanced stage of scurvy at the close of the experiment than those receiving orange extract similarly heated without the enzyme. The authors have at present no suggestion as to the significance of this fact.

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## THE EFFECT OF CERTAIN STIMULATING SUBSTANCES ON THE INVERTASE ACTIVITY OF YEAST.

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(Received for publication, August 2, 1921.)

The remarkable effect on yeast growth produced by the addition of an alcohol or water extract of yeast to a synthetic medium has been noted by several investigators. Wildiers<sup>1</sup> in 1901 first observed this fact. More recently others have confirmed Wildier's observations.

Euler<sup>2</sup> in studying invertase formation in yeast, found that the "Generationsdauer"—that is, the time required for yeast cells to double in number—was only one-half as long when a yeast extract was added to the nutrient medium as when other sources of nitrogen were used. Equivalent amounts of asparagine, glycocoll, alanine, cystine, and tyrosine were added singly or in combination, and all proved less favorable for yeast growth than the yeast extract.

Williams<sup>3</sup> presented evidence that the constituent of yeast which stimulates reproduction of yeast cells is identical with the antineuritic vitamine, and that the rate of reproduction of yeast cells might be used as a quantitative method for determining this substance. Almost simultaneously, Abderhalden and Koehler,<sup>4</sup> using the same method—microscopic observation of the growth of single yeast cells in hanging drops—found that the addition of dilute extracts of yeast markedly accelerated the rate of reproduction. Similar results were obtained with two other organisms, *Colpoda cucullus* and the alga, *Ulothrix*. These authors also

<sup>1</sup> Wildiers, E., *La Cellule*, 1901, xviii, 313.

<sup>2</sup> Euler, H., *Biochem. Z.*, 1918, lxxxv, 406.

<sup>3</sup> Williams, R. J., *J. Biol. Chem.*, 1919, xxxviii, 465.

<sup>4</sup> Abderhalden, E., and Koehler, A., *Arch. ges. Physiol.*, 1919, clxxvi, 209.

suggest that the active substance is the antineuritic vitamine and that yeast or some other simple organism might be used to determine its presence.

It is reasonable to suppose that a substance so potent in its effect on higher organisms as the antineuritic vitamine is known to be, must also play a significant rôle in the life processes of the yeast cell itself. The effect of the presence or absence of such a necessary substance on a simple cell like yeast can be more easily studied than in the more complex forms of life, and a knowledge of its function in the yeast cell undoubtedly is of fundamental importance for an understanding of its mode of action in higher organisms.

Abderhalden and Schaumann<sup>5</sup> have suggested the significance of this phase of yeast nutrition. They prepared an active extract by treating yeast with 10 per cent  $\text{H}_2\text{SO}_4$  for 24 hours. After filtering and removing the  $\text{H}_2\text{SO}_4$  with barium the solution was evaporated to dryness and the residue extracted with absolute alcohol five times. The combined alcohol extracts were evaporated under reduced pressure and the residue again extracted with absolute alcohol. This process was repeated until the residue was entirely soluble in alcohol. The alcohol solution was finally evaporated and the residue taken up in water. Such a yeast extract contained 8.95 per cent dry substance, 0.87 per cent ash, 0.19 per cent nitrogen, and 0.093 per cent phosphorus. Additions of 5 to 10 cc. of this extract to 250 cc. of a sugar solution in which living yeast was suspended accelerated greatly the fermentation of glucose, fructose, galactose, sucrose, and maltose. This action seemed to be specific for some unknown substance in the yeast extract in that amino-acids and phosphorus compounds, added to the fermentation mixtures, were entirely inactive. The  $\text{H}^+$  concentration was kept constant so as to eliminate that as a factor in the increased rate of fermentation.

A more highly purified preparation which they call "Eutonin" was precipitated from the alcohol extract by acetone. This was entirely phosphorus-free, but was still effective in augmenting fermentation by living yeast, although it was not so active as the original extract. These results indicate that in the "crude" extract more than one substance was active.

<sup>5</sup> Abderhalden, E., and Schaumann, H., *Fermentforschung*, 1919, ii, 120.

These questions arise: To what is the action of the yeast extract due? Does its effect lie solely in accelerating cell reproduction? Does it stimulate the formation of one or more of the enzymes? Or, finally, does it act directly on the enzymes as an activator or as a coenzyme?

Abderhalden and Schaumann state that the addition of yeast extract had some effect in accelerating fermentation when dried yeast or a maceration juice was substituted for the living yeast. On the basis of their results they conclude that there is a substance in the yeast extract which acts as a coenzyme or activator of an enzyme. However, their protocols show that in the experiments in which dried yeast or maceration juice was used, only slight increases in the rate of fermentation followed the addition of the yeast extract. As the determination of loss in weight was the method used for measuring the rate of fermentation the small increases in fermentation which were found in these experiments might reasonably come within the limits of experimental error. Also, there was opportunity for bacterial growth during the long period in which these mixtures were allowed to ferment without the addition of toluene.

When living yeast was used there was undoubtedly a remarkable increase in the rate of fermentation with the addition of yeast extract to the fermentation mixture. This increase occurred, too, after the tenth hour, during the period of most rapid growth of yeast.<sup>6</sup> It appears then that the chief effect of the active substance in yeast extract was on the living yeast, either in promoting growth or in stimulating the production of one or more of the enzymes.

Euler<sup>7</sup> and other workers have reported the variations in invertase activity of yeast grown in different media. Not only the substrate, sucrose, and the reaction products, glucose and fructose, but other hexoses as well, especially mannose, increase the formation of invertase.

Euler<sup>3</sup> also investigated the effect of nitrogen nutrition on invertase formation in yeast. He used  $(\text{NH}_4)_2\text{SO}_4$ , asparagine, glycocoll, alanine, tyrosine, and cystine as sources of nitrogen,

<sup>6</sup> Euler, H., and Lindner, P., *Chemie der Hefe und der alkoholischen Gärung*, Leipsic, 1915, 254.

<sup>7</sup> Euler, H., and Cramér, H., *Biochem. Z.*, 1913-14, lviii, 467.

the amino-acids having been added to the medium both singly and in combination. All these nitrogen compounds were about equal in their effect on growth and on the invertase activity of the yeast. However, when an equivalent amount of a water extract from yeast was used as a source of nitrogen, there was a decided increase both in the rate of growth and in the formation of invertase. Euler ascribes this effect to a nitrogenous substance in the yeast extract which is in the most available form for yeast nutrition. But here again it is obvious that some other stimulating substance present in the yeast extract may be responsible for these results.

The experiments reported in this paper were undertaken prior to the writer's knowledge of the Abderhalden work, and were devised to obtain further information concerning the effect of yeast extract on the enzymes of yeast. To begin with, invertase was chosen as the enzyme, the rate of formation of which was to be followed, because its activity is so easily measured and because it is so important in yeast action.

As the work progressed the question soon came up as to whether the substance which promotes growth of yeast also accelerates invertase formation. Yeast was grown with and without addition of solutions known to contain the growth stimulant. Various methods were used to separate this substance from other constituents and these partially purified preparations were also added to the standard medium. The yeast grown in these different media was filtered off and the invertase activity thereof determined.

#### EXPERIMENTAL.

##### *Method for Determining Invertase in Small Amounts of Yeast.*

For this work it was first necessary to devise a method for extracting invertase quantitatively from small amounts of yeast. Euler's method was as follows.<sup>2</sup> The filtered yeast was drained a few minutes on a porous plate, 0.25 gm. portions were weighed out and suspended in 10 cc. of 1 per cent  $\text{NaH}_2\text{PO}_4$  solution, and after standing 10 minutes this suspension was added to 20 cc. of 20 per cent cane-sugar solution. At stated times samples were removed to determine the rotation. The inversion was checked

and multirotation accelerated by adding 10 cc. of 5 per cent NaOH solution. Lövgren<sup>8</sup> used practically the same method.

A serious objection to this method is that the yeast cells are not killed and when inversion was allowed to continue several hours some growth and fermentation might occur. To obviate this, various methods of killing the yeast cell and extracting the enzyme were tried. Small amounts of yeast, 0.1 to 0.3 gm., were dried at 37° for 2 to 4 hours, then shaken with water to which a few drops of toluene were added. Other samples were ground in a mortar with fine sand or other abrasive material before treating with water. None of these methods gave uniform results.

Finally the method of Willstätter, Oppenheimer, and Steibelt<sup>9</sup> for the quantitative determination of maltase was adopted for our purpose. Fresh yeast was shaken with water and toluene. Willstätter and coworkers obtained almost complete extraction of maltase in 24 hours. There was a slight increase by 48 hours treatment. Because maltase is rapidly destroyed by acid, it was necessary to keep the extract neutral by frequent additions of  $\text{NH}_4\text{OH}$ . With invertase this was unnecessary since this enzyme is more resistant to acid and is also most active in faint acidity.

Invertase is more readily extracted by this method than is maltase. Fresh samples of Fleischmann's yeast were weighed out, suspended in 25 cc. of water, 0.5 cc. of acid-free toluene was added, and the mixtures were shaken moderately at 30° for 4, 8, 12, and 24 hours respectively. At the end of the stated time each mixture was transferred to a 100 cc. volumetric flask. 50 cc. of 20 per cent sucrose solution, 0.4 cc. of 0.1  $\text{N}$  HCl, and water to fill to the mark, were added. This was put into a 500 cc. Erlenmeyer flask and mixed well. 50 cc. were then removed, and 2 drops of  $\text{NH}_4\text{OH}$  (sp. gr. 0.90) added to check invertase action and to hasten multirotation. Before filtering it was necessary to add a small amount of talcum in order to obtain a perfectly clear filtrate. The rotation of the clear filtrate was then read in a 2 dm. tube at 20–25°C.

<sup>8</sup> Lövgren, S., *Fermentforschung*, 1919–20, iii, 221.

<sup>9</sup> Willstätter, R., Oppenheimer, T., and Steibelt, W., *Z. physiol. Chem.*, 1920, cx, 232.

The remaining 50 cc. were moderately shaken at 30°C. for 7 hours, after which the solution was made alkaline, filtered, and the rotation noted in the same way.

The results are given in Table I. It is evident that most of the activity was obtained by a preliminary treatment of 4 hours. There was a slight increase up to 12 hours, but a marked diminution in 24 hours.

TABLE I.

Weight of Fleischmann's yeast taken.	Time of shaking.	Change in $\alpha$ in 7 hrs.
<i>gm.</i>	<i>hrs.</i>	
0.2	4	11.88
0.2	8	11.84
0.2	12	12.38
0.2	24	8.36
0.3	4	14.90
0.3	8	14.98
0.3	12	15.24
0.3	24	12.71

As a final check on the method the invertase activity was determined on samples of yeast which were weighed out by another person. These results are found in Table II.

TABLE II.

Weight of Fleischmann's yeast taken.	Time of shaking.	Change in $\alpha$ in 7 hrs.
<i>gm.</i>	<i>hrs.</i>	
0.15	12	8.7
0.2	12	12.36
0.3	12	15.26

For the subsequent determinations the yeast was shaken with water and toluene at 30°C. for 12 hours—from 8.00 p.m. to 8.00 a.m.—then added to the sugar solution, and the change in rotation taken at the end of 7 hours. The toluene prevented any bacterial action.

It was found that the invertase extract was optically inactive. For convenience in taking the initial reading a blank was made up of 50 cc. of a 20 per cent sucrose solution plus 0.2 cc. 0.1 N HCl and water to make 100 cc. The rotation of this solution



was immediately taken for the initial reading. In the earliest studies the solutions were brought to a temperature of 20° before the rotations were read. The differences obtained in the readings with different samples of yeast were so great, however, that the slight errors due to a difference of 2 or 3° temperature were insignificant. Subsequent determinations were made at room temperature, which varied from 22–25°.

*Changes in Invertase Activity of Yeast Grown with the Addition of an Alcohol Extract of Yeast.*—An alcohol extract of yeast was prepared as follows: 3 pounds of starch-free Fleischmann's yeast were broken into fine pieces and dried in a current of warm air for 48 hours. The dry yeast was then covered with ether and heated on a water bath under a reflex condenser. This was repeated three times. Five extractions with 70 per cent alcohol were made in the same way. The alcohol extraction was continued over 2 days. The combined alcohol extracts were equal to 1,850 cc.

200 cc. of this alcohol extract were evaporated on the steam bath and the residue extracted with warm water four times. There was considerable lipin material present, so that in order to obtain a clear filtrate the water extract was run through a Berkefeld filter. The filtrate was made up to 200 cc. in water and labeled Alcohol Extract I.

Portions of this preparation were added to the medium in which yeast was grown to determine whether such an addition actually does increase the invertase activity of the yeast.

The medium used for the growth of the yeast was that adopted by Williams. It contained per liter

20.0	gm. of	sucrose.
1.5	" "	asparagine.
3.0	" "	(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> .
2.0	" "	KH <sub>2</sub> PO <sub>4</sub> .
0.25	" "	CaCl <sub>2</sub> .
0.25	" "	MgSO <sub>4</sub> .

500 cc. portions of this medium were introduced into each of four 2 liter conical flasks. This exposed a large surface and insured sufficient oxygen for vigorous growth. To two of the flasks were added 2.5 cc. of the alcohol extract plus 2.5 cc. of water, and to the other flasks 5 cc. of the same alcohol extract. These mixtures were sterilized at 10 pounds pressure for 15 minutes.

A yeast suspension was prepared by shaking 0.2 gm. of Fleischmann's yeast in a liter of distilled water. 5 cc. of this suspension were then introduced into each of the flasks with a sterile pipette. The flasks were then incubated at 30° for 24 hours. It was evident that growth was much greater in the flasks to which 5 cc. of alcohol extract had been added.

The yeast was then filtered by suction, onto alundum crucibles, washed twice with distilled water, and sucked dry. To facilitate the filtering, the contents of the flasks were first centrifuged and

TABLE III.

*Changes in Invertase Activity of Yeast Grown with the Addition of Alcohol Extract I. 0.2 Gm. (Moist Weight) Used in Each Invertase Test.*

Yeast grown with addition of extract.	Change in $\alpha$ in 7 hrs.
cc.	
2.5	4.31
	4.54
5	8.91
5	9.25
	9.73
2.5	5.38
	5.50
2.5	4.51
	4.36
5	7.08
	6.93
5	7.38
	6.79

most of the supernatant liquid was poured off. The yeast was then washed into the crucibles.

0.2 gm. portions of each sample of yeast were weighed out in duplicate, washed into Erlenmeyer flasks with 25 cc. of water, 0.5 cc. of toluene was added, and the mixtures were shaken 12 hours. The invertase preparation was then combined with the 20 per cent sucrose solution, 0.4 cc. of 0.1 N HCl, and water to make 100 cc. The initial rotation and the rotation at the end of 7 hours were taken as described.

These results are given in Table III. There is no doubt that the alcohol extract not only increases the rate of growth, but

also the invertase activity per unit weight of yeast. In this case when twice the amount of alcohol extract was added to the medium the invertase activity was doubled. This experiment was repeated a second time with similar results. Dry weights of these yeast samples were not obtained. However, the differences in moisture content could not account for the great differences in invertase activity of the different preparations. In subsequent experiments dry weights were determined and were found to be quite uniform in a single experiment.

The next step was to determine whether the substance which stimulates cell reproduction is identical with the substance which stimulates invertase formation. McCollum and Simmonds<sup>10</sup> have shown that the water-soluble vitamin is soluble in benzene after it has once been dissolved in alcohol. This suggested itself as a method of obtaining evidence as to whether the growth-promoting substance was also responsible for the increase in invertase formation.

*Invertase Activity of Yeast Grown with the Addition of Benzene-Soluble Material.*

200 cc. of the alcohol extract of yeast, equivalent to 2.74 gm. of total solid, were evaporated at 37° on 15 gm. of starch. This material was extracted with anhydrous ether for 6 hours to remove lipins. After this it was extracted with three portions of redistilled benzene (Kahlbaum's) for periods of 6, 7, and 8 hours. These combined benzene extracts were evaporated on a water bath and the residue was extracted with 200 cc. of water. This was called Benzene Extract I. It contained only 0.019 per cent of total solids and 0.0014 per cent of nitrogen. Yet when compared with Alcohol Extract I it was shown to be fully one-half as active in promoting growth (see Table IV).

5 and 10 cc. portions of the aqueous solution of this benzene extract were added to 500 cc. of the standard medium and 5 cc. of a yeast suspension introduced. The 24 hour growth of yeast was tested for its invertase activity as described above. The results are found in Table V.

<sup>10</sup> McCollum, E. V., and Simmonds, N., *J. Biol. Chem.*, 1918, xxxiii, 55.

TABLE IV.

*Comparison of Growth-Promoting Activity of Various Preparations.*

Extract used.	Growth of yeast cells per 1 cc. added to 30 cc. of medium.
Alcohol Extract I.....	469)
“ “ I.....	424) 446
Benzene Extract.....	198)
“ “ .....	230) 214
Alcohol Extract of benzene in soluble material.....	212)
“ “ “ “ “ “ “ .....	190) 201
Fullers' Earth Filtrate.....	34)
“ “ “ .....	28) 31
“ “ Extract.....	91)
“ “ “ .....	107) 99
Alcohol Extract II.....	418)
“ “ II.....	550) 484
Phosphotungstic Acid Filtrate.....	80)
“ “ “ .....	94) 87
“ “ “ I.....	30 30
“ “ “ II.....	11)
“ “ “ II.....	12) 11
Wheat Germ Extract.....	482)
“ “ “ .....	492) 487

TABLE V.

*Addition of Benzene Extract of Alcohol-Soluble Material from Yeast.*

Yeast grown with addition of benzene extract.	Change in $\alpha$ in 7 hrs.	Solids in the moist yeast.
cc.		per ccn l
5	2.425	15.8
	2.405	
5	2.525	14.5
10	2.34	15.6
10	2.405	15.1
20	2.63	23.2
20	2.81	
20	2.55	

Here, contrary to the expected results, the addition of increasing amounts of benzene extract had no effect on the invertase activity, although the growth was comparable to the amount of extract added.

*Effect of Adding Material Soluble in Alcohol after Benzene Extraction.*—The residue after benzene extraction was reextracted with alcohol for 7 hours, and the residue from this alcohol extract dissolved in 200 cc. of water. This in turn was added to the medium and yeast grown in it. As seen by the data given in Table VI, the substance which increases invertase concentration in the yeast has been recovered in this solution. By the benzene extraction there has been a separation of the growth stimu-

TABLE VI.

*Addition of Material Soluble in Alcohol Following a Thorough Extraction by Benzene.*

Yeast grown with addition of extract.	Change in $\alpha$ in 7 hrs.	Solids in the moist yeast.
cc.		<i>per cent</i>
5	7.40	26.7
	7.37	
5	10.83	26.8
	11.59	
20	16.93	25.1
	16.85	
20	16.89	25.5
	16.98	

lant from the substance responsible for the formation of the enzyme.

To confirm these results two other methods of separating the two substances were tried—precipitation with phosphotungstic acid and shaking with fullers' earth.

*Separation by Fullers' Earth.*—300 cc. of the alcohol extract of yeast were evaporated on the water bath and the residue was extracted with 200 cc. of water. After filtering, 100 cc. of this solution were shaken for 1 hour with 20 gm. of fullers' earth (Eimer and Amend). The earth was filtered off and washed once with water. The filtrate was made up to 150 cc. and called Fullers' Earth Filtrate.

The fullers' earth was shaken with 100 cc. of saturated  $\text{Ba}(\text{OH})_2$  for 10 minutes. The barium was removed from the combined filtrates with  $\text{H}_2\text{SO}_4$ . After concentrating on the water bath  $\text{BaSO}_4$  was filtered off, washed well, and the filtrate made up to 150 cc. This was called Fullers' Earth Extract.

A comparison of the activity of the filtrate and extract in promoting growth (by the counting method) shows the latter to be much more active (see Table IV). But here again the rate of growth and the rate of invertase formation do not run parallel.

Yeast grown with addition of 15 cc. of the Earth Extract was found to have almost identically the same invertase activity as yeast grown in standard medium with addition of only 15 cc. of distilled water. The rate of growth, however, was very different in the two cases. On the blanks, to which water was added, the growth from six flasks was required to yield 0.37 gm. of moist yeast (0.1354 gm. of dry weight). The addition of 15 cc. of Earth Extract produced a growth of 0.67 gm. of moist yeast (0.1687 gm. of dry weight) in two flasks.

On the other hand the addition of 15 cc. of Earth Filtrate to 500 cc. of medium yielded 0.965 gm. of moist yeast (0.2576 gm. of dry weight) in three flasks as compared with 1.8 gm. of moist yeast (0.5292 gm. of dry weight), the growth in two flasks with the addition of 15 cc. of the original alcohol extract. Yet the invertase activity in these two samples of yeast is of a similar order, being even a little greater in the yeast grown with the addition of the Earth Filtrate (see Table VII).

*Separation by Phosphotungstic Acid Precipitation.*—Williams was able to precipitate with phosphotungstic acid the substance which promotes yeast growth. An attempt was made to repeat this experiment as a third method of showing that the growth-promoting substance is not identical with the one which affects invertase formation.

150 cc. of a water solution of Alcohol Extract II (prepared as described for Alcohol Extract I) was evaporated to 20 cc. This concentrated solution was acidified to 5 per cent with  $\text{H}_2\text{SO}_4$  and 50 cc. of a 20 per cent solution of phosphotungstic acid in 5 per cent  $\text{H}_2\text{SO}_4$  were added. This was an excess of about 20 cc. of phosphotungstic acid. It was allowed to stand in the ice box over night. The precipitate was then filtered off by suction and

washed twice with 10 per cent phosphotungstic acid in 5 per cent  $\text{H}_2\text{SO}_4$ .

The filtrate was freed from phosphotungstic acid by shaking in a Squibb separatory funnel with a mixture of equal parts of amyl alcohol and ether.  $\text{H}_2\text{SO}_4$  and the last traces of phosphotungstic acid were removed from the filtrate by adding a slight excess of  $\text{Ba}(\text{OH})_2$  and filtering. The precipitate was well washed and the filtrate made slightly acid with  $\text{H}_2\text{SO}_4$ , after which it was concentrated on the water bath to 150 cc., again neutralized and filtered. This was called Phosphotungstic Acid Filtrate.

The precipitates were suspended in 75 cc. of 5 per cent  $\text{H}_2\text{SO}_4$  and decomposed by shaking with the amyl alcohol-ether mixture.

TABLE VII.

*Separation of Growth Stimulant from Substance which Stimulates Production of Invertase by Means of Fullers' Earth.*

Yeast grown with addition of:	Change in $\alpha$ in 7 hrs.	Solids in the moist yeast.	
		per cent	
15 cc. Original Alcohol Extract I.....	6.39	27.5	29.4
	6.55	31.3	
15 " Earth Filtrate.....	7.63	26.9	26.8
	8.99	26.6	
15 " " Extract.....	3.23	25.5	25.1
	4.35	24.7	
15 " water.....	3.05	36.6	

Only part of the precipitates could be decomposed in this manner. The remaining precipitate was filtered off and decomposed in the usual manner by treating with saturated  $\text{Ba}(\text{OH})_2$  solution. The barium was then precipitated with  $\text{H}_2\text{SO}_4$ . The filtrate from the  $\text{BaSO}_4$  was diluted to 150 cc. and called Phosphotungstic Acid Precipitate II.

The acid solution of precipitates decomposed by amyl alcohol-ether treatment was neutralized with  $\text{Ba}(\text{OH})_2$  solution. This filtrate was made up to 150 cc. and called Phosphotungstic Acid Precipitate I.

Williams<sup>3</sup> found that he recovered from the phosphotungstic precipitate even greater activity in growth promotion than was

present in the original solution. He ascribes this to the effect of acid hydrolysis. The writer was unable to obtain such results. In two attempts to separate the growth stimulant by phosphotungstic precipitation a great loss in activity was observed (see Table IV). Also, the addition of the phosphotungstic acid filtrate produced greater growth than either one of the decomposed precipitates or even than the combined precipitates. As yet it is not known just where this loss of activity occurred.

There was no loss, however, in the substance which accelerated invertase formation. That remained almost entirely in the filtrate, as may be seen by referring to Table VIII.

*A gummy precipitate which separated from the original hot alcohol extract on cooling contained the substance active in invertase formation in high concentration.* A water solution made of a small amount of this precipitate was compared with Alcohol Extract II for activity both in promoting growth and in stimulating invertase formation. The data are given in Table IX. The solution of the precipitate although only one-tenth as active in increasing growth was equal in its effect on invertase formation.

This is further evidence that the active substance in promoting reproduction of yeast cells, whether it is the water-soluble vitamine or a stimulant specific for yeast, is not the constituent which affects the invertase concentration of the yeast.

*Alcohol Extract of Wheat Germ.*—Having found in yeast extract a substance so potent in accelerating invertase formation in yeast during growth, it seemed of interest to investigate extracts of other materials known to be rich in the growth stimulant. Thus far the only preparation we have used is an alcohol extract of wheat germ. 50 gm. of wheat germ were dried in a vacuum desiccator over  $\text{CaCl}_2$  for 3 days. This was extracted with ether for 6 hours, followed by extraction with alcohol for three periods of 6, 6, and 8 hours, using fresh portions of alcohol each time.

100 cc. of a water solution were made up equivalent to 15 gm. of the wheat germ. This was very active in promoting growth (see Table IV). Yet, addition of this preparation had apparently no effect on the invertase formation in the yeast. As seen by data given in Table X, increasing the amount of wheat germ extract by four times had no effect in stimulating greater invertase production.



TABLE VIII.

*Separation of Growth Stimulant from Substance Which Stimulates the Production of Invertase by Phosphotungstic Acid Precipitation.*

Yeast grown with addition of:	Change in $\alpha$ in 7 hrs.	Solids in the moist yeast.
		<i>per cent</i>
10 cc. Original Alcohol Extract II.....	5.44 5.51	26.9
10 " Filtrate from Phosphotungstic Precipitate .....	5.31 5.41	26.5
10 " Precipitate I.....	1.83 1.97	28.4
10 " " II.....	1.42 1.34	27.6
10 " water.....	0.57 0.49	26.8

TABLE IX.

Yeast grown with addition of:	Change in $\alpha$ in 7 hrs.	Solids in the moist yeast.	Growth of yeast cells per 1 cc.
		<i>per cent</i>	
10 cc. Alcohol Extract II.....	5.44	28.2 } 25.7 } 26.9	418 } 550 } 484
10 " solution of gummy precipitate.....	5.29 5.55	22.5 } 27.9 } 25.2	54 } 44 } 49

TABLE X.

*Alcohol Extract of Wheat Germ.*

Yeast grown with addition of:	Change in $\alpha$ in 7 hrs.	Solids in moist yeast.
		<i>per cent</i>
2.5 cc. Wheat Germ Extract.....	2.08 1.86 2.15	24.5 } 24.6 } 24.5
10 " " " " .....	2.26 2.27 2.37	24.5 } 24.3 } 24.4

*Addition of Alcohol Extract to an Invertase Preparation.*

As already stated Abderhalden and Schaumann<sup>5</sup> draw the conclusion that fermentation of sugars by dried yeast or by maceration juice is also accelerated by the addition of the yeast extract. If this is true some substance in the extract must act as a coenzyme or as an activator of a proferment.

This was found not to be true, however, in the case of invertase. An invertase extract was prepared by shaking 2 gm. of fresh Fleischmann's yeast with 100 cc. of water and 2 cc. of toluene for 12 hours. Solutions were then made up in duplicate as follows:

(a) 50 cc. of a 20 per cent sucrose solution, 0.4 cc. 0.1 N HCl, 25 cc. of invertase preparation and water to make 100 cc.

(b) The same as (a) except that 5 cc. of Alcohol Extract I were substituted for an equal amount of water.

(c) The same as (a) except that 5 cc. of Benzene Extract were substituted for an equal amount of water.

These were shaken at 30°C., portions being removed at the end of 2 and 4 hours, and the rotation was noted. The results show no acceleration of inversion by addition of these solutions, one of which, the alcohol extract, was very efficacious in stimulating invertase formation as well as growth. The results are given in Table XI.

TABLE XI

*Effect of Growth-Stimulating Substance on the Activity of the Enzyme.*

Material added:	Change in $\alpha$ in 2 hrs.	Change in $\alpha$ in 4 hrs.
5 cc. Alcohol Extract I.....	5.61	10.29
5 " " " I.....	5.75	10.60
5 " Benzene Extract.....	6.08	10.94
	6.00	10.80
5 " Water.....	5.95	10.89

This was repeated with a much less active invertase preparation with the same results. There was no possibility, therefore, that in the first case the invertase preparation itself contained sufficient of the stimulating substance to give the maximum effect.

## DISCUSSION.

It is evident from the results obtained in these experiments that some constituent of yeast is soluble in 70 to 95 per cent alcohol and still more soluble in water, which, when added in small amounts to a medium in which yeast is grown, will increase the invertase activity of such yeast to a remarkable extent. Contrary to expectation this substance seems to be something other than the growth-stimulating element. Euler considered that it was a nitrogenous substance which was easily available for yeast nutrition and which had this effect in accelerating invertase formation.

On the other hand, carbohydrates in the medium are known to cause an increase in the invertase activity of the yeast. This is especially true of mannose. Mathews and Glenn<sup>11</sup> found that active invertase preparations always contained about 70 per cent of a mannose gum. This suggests that the substance in yeast extract which has this specific effect in increasing the invertase activity of yeast is some form of carbohydrate. The fact that this activity was found in high concentration in the gummy precipitate which separated from the hot alcohol extract of yeast, is very suggestive in this connection. Further work is planned to identify the substance.

## SUMMARY.

1. The results of these experiments have confirmed the presence in a water or alcohol extract of yeast of a substance which accelerates the rate of invertase formation during a 24 hour period of growth.

2. This substance is not identical with the growth stimulant. A partial separation of the two substances has been accomplished by three methods: (a) Extraction of the growth stimulant with benzene, (b) adsorption with fullers' earth, and (c) precipitation with phosphotungstic acid.

3. The substance which accelerates invertase formation was found in high concentration in the gummy precipitate separated from an alcohol extract of yeast.

<sup>11</sup> Mathews, A. P., and Glenn, T. H., *J. Biol. Chem.*, 1911, ix, 29.

4. Extracts of wheat germ, very active in stimulating growth, did not increase the invertase concentration of yeast when added to the medium.

5. The yeast extract does not act directly upon the invertase itself. Therefore the substance does not appear to be of the nature of an activator or coenzyme.

In conclusion I wish to express to Professor F. C. Koch my appreciation of his generosity in giving helpful advice during the progress of this work.

# DETERMINATION OF THE MONOAMINO-ACIDS IN THE HYDROLYTIC CLEAVAGE PRODUCTS OF LACTALBUMIN.\*

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(Received for publication, August 25, 1921.)

During recent years a large advance has been made in the methods for the determination and isolation of the amino-acids yielded by proteins on hydrolysis. The recent discovery by Dakin (1) of hydroxyglutamic acid among the hydrolysis products of casein has also been a most valuable contribution in this field. It is very probable that the lists of amino-acids determined in many proteins during the earlier years should also include this amino-acid. The only published analysis of the mono-amino-acids in lactalbumin is that published in 1907 by Abderhalden and Přibram (2). On account of the greatly increased prominence given milk as of paramount importance in human nutrition it was thought to be of interest to make a new analysis of the products of hydrolysis of lactalbumin in the light of the newer methods. In this analysis only the monoamino-acids are determined, since an analysis of the diamino-acids has been made by the Van Slyke method by Osborne, Van Slyke, Leavenworth, and Vinograd (3).

The lactalbumin used in this hydrolysis was obtained in connection with the preparation of protein-free milk used in nutrition experiments. Fresh skimmed milk was heated to a temperature of 35°C. by passing in a current of steam. Normal hydrochloric acid was then added until a test portion of the whey showed a hydrogen ion concentration of approximately 4.6. The filtrate

\* A preliminary report of this work was presented at the fifteenth annual meeting of the Society of Biological Chemists held in Chicago, December 28 to 30, 1920 (cf. *J. Biol. Chem.*, 1921, xlv, p. xii).

from the precipitated casein was boiled for 10 minutes in a steam-jacketed kettle. The coagulated protein was washed several times with hot water and suspended in dilute alcohol. It was afterwards allowed to stand in absolute alcohol, then in anhydrous ether, and finally dried at 110°C. This preparation contained 5.85 per cent moisture, 0.91 per cent ash, and calculated ash- and moisture-free, 15.39 per cent of nitrogen (Kjeldahl).

*Hydrolysis of Lactalbumin.*

*Glutamic Acid.*—The hydrolysis was effected by boiling for 40 hours on an oil bath<sup>1</sup> 325 gm. of the lactalbumin, equivalent to 303 gm. of the ash- and moisture-free protein, with 1,450 cc. of hydrochloric acid (specific gravity 1.1). After diluting the hydrolysate somewhat, the suspended humin was removed by filtration. This amounted to 5.77 gm., and contained 7.66 per cent nitrogen. The nearly black filtrate was concentrated under reduced pressure to about 450 cc. which on cooling partly solidified. It was then redissolved in a small amount of water and saturated cold with hydrochloric acid. The crystalline product which had separated after standing at 0° for 5 days was redissolved, decolorized with norit, and resaturated with hydrochloric acid. Examination of a test portion of the glutamic acid hydrochloride thus obtained showed that it was practically free from ammonium chloride. It decomposed with effervescence at 199–200°, and gave 7.78 per cent of amino nitrogen as determined by the Van Slyke method (calculated, 7.65 per cent). There were thus isolated 29.42 gm. of glutamic acid hydrochloride equivalent to 23.57 gm. of the free acid, which, together with the 15.49 gm. subsequently obtained by the lime-alcohol method, amounts to 39.06 gm., or 12.89 per cent of the protein.

The united filtrates and washings from the glutamic acid hydrochloride were diluted with water to make a volume of about 12 liters, and hydrochloric acid was added in the amount neces-

<sup>1</sup> The material used for the oil bath consisted of a highly hydrogenated cottonseed oil, which fused at about 58–60°. We have used it for this purpose for a considerable time and have found it to be far superior to the materials commonly used for oil baths. Little or no odor or fumes are evolved when it is heated for long periods at temperatures as high as 170°.

sary to make the solution contain 3.5 per cent. The diamino-acids were then removed from the solution in the usual way by means of phosphotungstic acid and the excess of the latter removed quantitatively, first by means of ether and amyl alcohol and the remaining traces by means of barium hydroxide. The solution of monoamino-acids was treated with an excess of freshly slaked lime according to Foreman's lime-alcohol method (4) for the separation of the dibasic acids. After filtering off the excess of calcium hydroxide, the solution of calcium salts was concentrated under reduced pressure to about 1,000 to 1,200 cc. In all of the foregoing work the concentrating of solutions was done under reduced pressure and at a temperature not exceeding 40-42°, in order to avoid the formation of the pyrrolidone carboxylic acids from glutamic and hydroxyglutamic acids. The calcium salts of the dibasic acids were then precipitated in the usual way by the addition of alcohol, and the large amount of precipitate thoroughly washed with 95 per cent alcohol. This precipitate also contained considerable tyrosine, as will be shown in a subsequent paragraph. The dibasic acids were recovered by removal of the calcium with oxalic acid. The hydrochloric acid in the solution was then removed by means of silver acetate and the excess of silver with hydrogen sulfide. The solution of dibasic acids was concentrated at low temperature and, after filtering off 0.63 gm. of tyrosine, the filtrate was further concentrated until the amino-acids had separated in the form of a thick paste. This was removed from the flask as far as possible and dried *in vacuo* over powdered sodium hydroxide and calcium chloride; the parts adhering to the sides of the flask were washed out with small amounts of water and added to the main portion. The amino-acids, thus obtained and dried, weighed 87.93 gm. In order to separate the glutamic and aspartic acids from other products present the dry residue was triturated with glacial acetic acid. This residue was difficult to disintegrate with the acetic acid and formed a pasty mass which filtered very slowly. The portion remaining undissolved in the acetic acid was triturated two more times with fresh acetic acid. The disintegration of the mixture at first with acetic acid can be greatly facilitated by allowing the substance to soak in the acetic acid over night or longer and then thoroughly triturating in a mortar.

After filtering by suction the acid-insoluble substance was dried on the filter by drawing air through the suction flask and gradually reducing the lumps by means of a spatula. The nearly white powder thus obtained weighed 55.67 gm.

*Aspartic Acid*.—The above product was dissolved in about 2 liters of water and boiled with an excess of copper carbonate. After filtering off the excess of the latter the solution yielded 54.33 gm. of copper aspartate, which separated in the characteristic crystal form. This salt, after washing with cold water and drying in the air at room temperature, gave the following results on analysis:

Analysis I. 0.3387 gm. substance: 0.0974 gm. copper oxide.

$C_4H_5C_4NCu \cdot 4\frac{1}{2}H_2O$ . Calculated. Cu 23.07.

Found. Cu 22.98.

The filtrate from the copper aspartate on further concentration gave a pale blue copper salt, which after decomposition with hydrogen sulfide gave 2.79 gm. of pure glutamic acid. Copper was removed from the filtrate and from the copper glutamate and the concentrated solution, after saturating with hydrochloric acid, yielded 9.94 gm. of glutamic acid as the hydrochloride. The filtrate from the glutamic acid hydrochloride, after removal of hydrochloric acid by distillation and finally with silver sulfate, gave 1.96 gm. of aspartic acid in the form of the copper salt. The total amount of aspartic acid isolated was 28.18 gm., which represents 9.30 per cent of the protein.

The filtrate from the last crop of copper aspartate, after removal of the copper with hydrogen sulfide, was concentrated, and by removal of successive crops of crystals and fractional crystallization there were obtained 2.16 gm. of tyrosine and 2.76 gm. of glutamic acid. The final filtrate gave a syrup from which nothing definite could be isolated. This syrup still gave a strong positive test for tyrosine with Millon's reagent. It is of interest to note that 2.79 gm. of tyrosine, which is equivalent to 47.3 per cent of the total amount of tyrosine obtained from the protein hydrolyzed, were isolated from the dibasic acid fraction of the calcium salts which were obtained by the precipitation with alcohol.

*Hydroxyglutamic Acid*.—The acetic acid extracts remaining after triturating the product obtained from the precipitated



calcium salts were concentrated at a temperature not over 40° to a thin syrup. After standing for several weeks over calcium chloride and powdered sodium hydroxide, a thick viscous syrup was obtained. Analyses showed that it contained 2.8780 gm. of total nitrogen and 2.7115 gm. of amino nitrogen. This amount of amino nitrogen, if due entirely to hydroxyglutamic acid, represents 31.6 gm. of this acid, which is a little over 10 per cent of the protein. Subsequent careful examination of this substance indicated that the above figure represents quite closely the amount of hydroxyglutamic acid that was present in this fraction. The acid was then made nearly neutral by addition of sodium carbonate, and mercuric acetate added until no further precipitation of the white salt occurred. After thoroughly washing the precipitate with water it was decomposed with hydrogen sulfide. The filtrate from the mercuric sulfide was concentrated at 40° to about 40 to 50 cc., and a relatively large amount of absolute alcohol added. The oily product which separated was washed several times with fresh absolute alcohol. Under the treatment with absolute alcohol this product gradually hardened and was finally obtained in a coarsely granular form. It was then dried, at first *in vacuo* over calcium chloride, and finally over phosphorus pentoxide at room temperature at 16 mm. pressure. The acid thus obtained was very hygroscopic. On heating at about 60° it appeared to soften, and at about 85° it became pasty and gradually intumesced, leaving an opaque column. It did not materially change in appearance on further heating until a temperature of about 140° was reached. Between 140 and 150° it changed, with slight effervescence, to a clear liquid. This behavior on heating corresponds quite closely to that given by Dakin for hydroxyglutamic acid. A sample on analysis gave 38 per cent carbon. This figure is about 1 per cent too high for that required for hydroxyglutamic acid (36.81 per cent). This discrepancy was most likely due to the presence of a little tyrosine, as a distinctly positive test for tyrosine was obtained with Millon's reagent. On account of the high carbon content of tyrosine (59.67 per cent), only a small amount of this acid would be required to account for the above high result.

For further purification the acid was dissolved in a small amount of water, reprecipitated with alcohol, and the oil which separated

thoroughly washed with several fresh portions of alcohol. After drying, first with absolute alcohol, and finally with calcium chloride and phosphorus pentoxide *in vacuo* as above described it was analyzed with the following results:

Analysis 2. 0.2707 gm. substance: 0.3655 gm. carbon dioxide and 0.1357 gm. water.

$C_5H_9O_5N$ . Calculated. C 36.81, H 5.52.

Found. C 36.82, H 5.61.

A portion of the acid was made nearly neutral with potassium hydroxide and converted into the silver salt by alternate addition of silver nitrate and potassium hydroxide.

Analysis 3. 0.3017 gm. substance: 0.2296 gm. silver chloride.

$C_5H_7O_5NAg_2$ . Calculated. Ag 57.26.

Found. Ag 57.27.

*Alcoholic Filtrate from the Precipitated Calcium Salts.*

*Tyrosine.*—The alcoholic filtrate from the calcium salts of the dicarboxylic acids, which amounted to 5 to 6 liters was concentrated to 800 to 900 cc., and the calcium removed with ammonium oxalate. The filtrate from the calcium oxalate was concentrated and two crops of crystals were successively removed. These weighed about 8 gm., and on fractional crystallization yielded 2.89 gm. of tyrosine. This, together with the 2.79 gm., which were precipitated with the calcium salts of the dicarboxylic acids already referred to, and 0.22 gm. subsequently isolated from the barium residues of the unesterified amino-acids, amount to 5.90 gm. or 1.95 per cent of the protein. A representative sample of the tyrosine isolated gave the following results on analysis:

Analysis 4. 0.2985 gm. substance required 16.2 cc. of 0.1 N acid.

$C_9H_{11}O_3N$ . Calculated. N 7.73.

Found. N 7.62.

A complete separation of tyrosine from the other products of protein hydrolysis is extremely difficult to accomplish. This is shown by the fact that some tyrosine was either isolated, or shown to be present, by microscopic examination and tests with Millon's reagent, in most of the main fractions of this hydrolysis.

It was partly precipitated by alcohol with the calcium salts of the dicarboxylic acids, a considerable amount was obtained from the alcoholic filtrate from the calcium salts and a small amount isolated from the barium residue of the unesterified fraction. Furthermore, some was esterified and later detected in the distillation residue. Tyrosine, however, is esterified with difficulty by means of the lead salt method of esterification. Although pure tyrosine is one of the least soluble of the amino-acids obtained from proteins, nevertheless its presence has been frequently detected in residual filtrates from which such soluble amino-acids as alanine, glycine, and serine have been removed as far as possible. The percentage of tyrosine isolated as given above must therefore be regarded as minimal.

*Proline.*—The main solution of amino-acids containing the filtrates and washings from the tyrosine were concentrated to a small volume and the ammonium chloride, which had formed when removing the calcium with ammonium oxalate, was decomposed, and the ammonia expelled by boiling with a slight excess of barium hydroxide. Barium was removed and the recovered, free, dry amino-acids extracted with boiling absolute alcohol. Proline was determined according to the method described in a previous publication from this laboratory (5). Two other proline determinations made on separate hydrolysis of 10 gm. samples of the protein gave closely agreeing results. The average of all of the results obtained was 3.76 per cent.

For the separation of the amino-acids remaining after the removal of proline by extraction with alcohol, they were converted into their ethyl esters according to Foreman's method (6). For this purpose an excess of lead oxide was suspended in the solution of amino-acids and steam passed in for about 45 minutes. After filtering off and washing the excess of lead oxide the solution was evaporated to dryness, and the 216 gm. of dry lead salts obtained were suspended in absolute alcohol and the mixture was saturated at a temperature below 0° with dry hydrochloric acid gas. The greater part of the hydrochloric acid in the filtrate from the lead chloride was removed by distillation. The solution was then placed in a freezing mixture and nearly neutralized by addition of absolute alcohol which had been saturated with dry ammonia. The precipitated ammonium chloride was filtered

off and the alcohol removed by distillation under reduced pressure. The residue was dissolved in dry chloroform and the esters were liberated by means of dehydrated barium hydroxide. After removal of the excess of barium hydroxide by filtration, and the chloroform by distillation, the residual esters were dissolved in anhydrous ether and the ethereal solution was allowed to stand for several days with anhydrous sodium sulfate. There were obtained, after distilling off the ether, 149 gm. of esters.

*Glycine.*—The esters which were carried over by the vapors during the removal of the alcohol and chloroform by distillation were recovered and added to the final ether distillate from the esters. The solution was strongly acidified with alcoholic-hydrochloric acid, and allowed to stand at 0° for nearly a month. About 0.2 gm. of a white crystalline substance was filtered off. This melted to a clear oil at 138–140° indicating that it was slightly impure glycine ester hydrochloride. This was added to the residue obtained after the removal of the ether by distillation. Hydrochloric acid was quantitatively removed from the residue by means of silver sulfate, the silver with hydrogen sulfide, and the sulfuric acid with barium hydroxide. The residue remaining after evaporating the solution to dryness weighed 1.19 gm. To this was added the 0.92 gm. obtained by evaporating the final filtrate from the alanine from Fraction I of the distilled esters. From this mixture there were obtained in the usual way 2.83 gm. of glycine picrate, which decomposed at 200° (uncorrected). (Levene and Van Slyke (7) state that it softens at 199–200°, and decomposes at 202°.) The above amount of glycine picrate is equivalent to 1.12 gm. of glycine, or 0.37 per cent of the protein.

*Serine.*—The barium residues remaining after filtering the chloroform solution of the esters were decomposed with warm dilute sulfuric acid, and the barium sulfate washed until the washings gave no test for tyrosine. Hydrochloric acid was removed with silver sulfate and the sulfuric acid with barium hydroxide. The solution was then concentrated and after removal of 0.22 gm. of tyrosine the remaining amino-acids were fractionally crystallized. A fraction was obtained which consisted chiefly of serine but also contained, besides some tyrosine, a small amount of a substance which gave to the solution of the mixture a decided

acid reaction. Extensive fractional crystallization failed to effect the separation of the serine from the admixed impurities. The solution was finally neutralized by addition of a few drops of ammonium hydroxide. On slow evaporation crystals of tyrosine separated. These were removed and from the filtrate 5.34 gm. of serine crystallized in large compact plates. The serine had a decidedly sweetish taste and on heating darkened at 210–215° and decomposed with effervescence at 232–233° (uncorrected). Analysis<sup>2</sup> showed it to have the following composition:

Analysis 5. 0.1914 gm. substance: 0.2411 gm. carbon dioxide and 0.1192 gm. water.

$C_3H_7O_3N$ . Calculated. C 34.29, H 6.67.  
Found. C 34.35, H 6.97.

The esters remaining after the distillation of the ether were separated into the following fractions by distillation under reduced pressure:

TABLE I.

Fraction No.	Temperature of the bath up to.	Temperature of the vapors up to.	Pressure.	Weight.
	°C.	°C.	mm.	gm.
I.....	80	65	13	12
II.....	115	100	2	80
Distillation residue.....				16
Contents of the liquid air tube ....				22

*Fraction I. Alanine and Valine.*—To this fraction were added the contents of the liquid air tube which contained besides esters some ether and chloroform. After hydrolyzing the esters by boiling with water for 7 to 8 hours, the solution was concentrated under reduced pressure and six crops of crystals were successively removed. Absolute alcohol was added to the filtrate from the last crop and the precipitated amino-acids filtered off. The final filtrate was evaporated to dryness and the residue together with the crop which was precipitated by alcohol added to the amino-acids which were examined for glycine. By fractional crystallization from water and from water and alcohol

<sup>2</sup> The carbon and hydrogen determinations recorded in this hydrolysis were made by Mr. C. E. F. Gersdorff.

there were obtained 7.29 gm. of alanine, and a small amount of a mixture of leucine and valine from which were isolated, by means of the lead salt method of Levene and Van Slyke (8), 1.37 gm. of leucine and 1.37 gm. of valine. Analysis of the alanine gave the following results.

Analysis 6. 0.1612 gm. substance: 0.2373 gm. carbon dioxide and 0.1161 gm. water.

$C_3H_7O_2N$ .	Calculated.	C 40.45, H 7.86.
	Found.	C 40.15, H 8.06.

*Fraction II. Leucine and Valine.*—After hydrolysis of the esters of this fraction in the usual way the aqueous solution of amino-acids was concentrated under reduced pressure, and eleven crops of amino-acids were obtained by their successive removal as they separated out in the distillation flask. The first three crops weighing 28.11 gm. were practically pure leucine and 1.38 gm. were further obtained by fractional crystallization of the fourth crop.

Analysis 7. 0.1813 gm. substance: 0.3657 gm. carbon dioxide and 0.1565 gm. water.

$C_6H_{13}O_2N$ .	Calculated.	C 54.96, H 9.99.
	Found.	C 55.01, H 9.66.

Further fractional crystallization and carbon and hydrogen determinations showed that the remaining acids of this fraction consisted of considerable valine. Use was made of the lead salt method of separation and 17.61 gm. of the lead salt of leucine (equivalent to 9.88 gm. of leucine) were isolated. The free leucine obtained by decomposition of the lead salt with hydrogen sulfide gave on analysis 54.91 per cent carbon, and 9.96 per cent hydrogen.

The amino-acids remaining in the filtrate from the lead leucine were recovered in the usual way. Difficulty was encountered in isolating the valine in a pure form on account of the presence of some substance which had strong acidic properties. Subjecting of the mixture to the lime-alcohol method for removing the dicarboxylic acids proved to be inadequate for the complete removal of this acid, although a very small amount of a syrup was obtained from the calcium salts precipitated by the alcohol.

This syrup was insoluble in alcohol, strongly acidic, and could not be brought to crystallization. On heating with zinc dust it gave a strong pyrrole test. Lack of material prevented any further examination of this substance. The only explanation which we can advance at present as to its character and presence in the leucine fraction is that not all of the hydroxyglutamic acid was precipitated together with the other dicarboxylic acids as the calcium salts, and that this portion which escaped precipitation was subsequently esterified and that some of its ester distilled over with the leucine and valine esters of Fraction II.

The mixture of amino-acids was recovered from their calcium salts and recrystallized from water, after having first made the solution faintly alkaline with ammonium hydroxide. In this way most of the crystalline amino-acids were obtained free from the acid impurity, and after removal of 1.73 gm. of leucine as the lead salt 8.63 gm. of fairly pure valine were obtained.

Analysis 8. 0.1885 gm. substance: 0.3549 gm. carbon dioxide and 0.1586 gm. water. 0.1400 gm. substance required 11.8 cc. of 0.1 N sulfuric acid.

$C_5H_{11}O_2N$ .	Calculated.	C 51.28, H 9.47, N 11.96.
	Found.	C 51.35, H 9.42, N 11.83.

*Phenylalanine*.—The residue remaining after the distillation of the esters was shaken with water and ether in the usual way in order to remove the phenylalanine ester. The ester extracted by the ether was dissolved in concentrated hydrochloric acid and the solution evaporated to 15 to 20 cc. on a steam bath. From this solution there were obtained 3.75 gm. of phenylalanine in the form of the hydrochloride. The free phenylalanine obtained by decomposition of the hydrochloride with ammonium hydroxide gave the following results on analysis:

Analysis 9. 0.1421 gm. substance: 0.3418 gm. carbon dioxide and 0.0838 gm. water.

$C_9H_{11}O_2N$ .	Calculated.	C 65.45, H 6.66.
	Found.	C 65.60, H 6.60.

The aqueous solution remaining after removing the phenylalanine ester with ether was boiled with barium hydroxide and the barium quantitatively removed with sulfuric acid. Aside from a small amount of crystals, which both from appearance and

composition indicated leucine, nothing of a definite character could be isolated.

#### DISCUSSION.

The results of the preceding analysis of the products formed by the hydrolysis of lactalbumin are collected in Table II, together with those obtained by Abderhalden and Přibram. The percentages of the diamino-acids obtained by Osborne, Van Slyke, Leavenworth, and Vinograd, as determined by the Van Slyke method, are also given and added to those of the monoamino-acids determined in this analysis. In comparing our results with those of Abderhalden and Přibram the most striking differ-

TABLE II.  
*Percentage of Amino-Acids in Lactalbumin.*

Amino-acid.	Jones and Johns.	Abderhalden and Přibram.
Glycine.....	0.37	.
Alanine.....	2.41	2.5
Valine.....	3.30	0.9
Leucine.....	14.03	19.4
Proline.....	3.76	4.0
Phenylalanine.....	1.25	2.4
Aspartic acid.....	9.30	1.0
Glutamic acid.....	12.89	10.1
Hydroxyglutamic acid.....	10.00	
Serine.....	1.76	
Tyrosine.....	1.95	0.85
Total monoamino-acids.....	61.02	41.15
Cystine.....	1.73*	
Arginine.....	3.47*	
Histidine.....	2.61*	
Lysine.....	9.87*	
Tryptophane.....	Present.	
Ammonia.....	1.31*	
Total diamino-acids and ammonia.....	18.99	18.99*
Total.....	80.01	60.14

\* Osborne, T. B., Van Slyke, D. D., Leavenworth, C. S., and Vinograd, M., *J. Biol. Chem.*, 1915, xxii, 266.



ence, aside from the hydroxyglutamic acid, is shown by the figures for aspartic acid, the percentage of this amino-acid found in this hydrolysis being over nine times that previously recorded. It is generally recognized by investigators who are familiar with the details of the analysis of the products of protein hydrolysis that the determination of aspartic acid as made by the ester method of Emil Fischer is very uncertain. Osborne and Jones (9) in a study of the consideration of the sources of loss in analyzing the products of protein hydrolysis were able to recover only 42.5 per cent of the aspartic acid which was used in a mixture of amino-acids after these amino-acids had been subjected to the same treatment as employed in the hydrolysis of proteins and the analysis of their degradation products by means of the Fischer method. The difficulty in the way of obtaining better results in the determination of aspartic acid by the Fischer method has been due to several causes: Incomplete esterification, losses through decomposition of the esters during distillation, and incomplete saponification with the formation of the half ester. The chief cause, however, has been undoubtedly due to the fact that the aspartic acid was often associated with considerable quantities of pyrrolidone carboxylic and hydroxyglutamic acids. These acids form syrupy mixtures from which the aspartic acid would crystallize very slowly if at all; the same would correspondingly apply to their copper salts. By means of the newer methods used in this hydrolysis these difficulties have been entirely avoided. The new result for valine is considerably higher, while the difference in the combined values for valine and leucine is not great. This higher figure for valine is probably due to a more complete separation of this acid from the leucine, which was made possible by means of the lead salt method of Levene and Van Slyke (8). The percentages of alanine, proline, and glutamic acid are in fairly close agreement. Our figure for tyrosine, although twice that found by Abderhalden and P'ibram, is without question too low, since evidences of tyrosine were found in several fractions, from which it could not be isolated in pure enough condition to be weighed. The considerable amount of hydroxyglutamic acid found is of interest, especially so inasmuch as Dakin (1) first discovered this amino-

acid in about the same percentage in casein. This makes the total proteins of milk particularly high in this most recently discovered amino-acid constituent of proteins.

#### SUMMARY.

The lactalbumin was prepared from fresh skim milk. The casein was first precipitated at 35° by normal hydrochloric acid at a hydrogen ion concentration of 4.6. The lactalbumin obtained by boiling the filtrate from the casein for 10 minutes, was dried with alcohol and ether, and finally at 110°.

The protein was hydrolyzed by boiling for 40 hours with hydrochloric acid (specific gravity 1.1) and the resulting monoamino-acids determined, use being made of the more recent methods for their separation.

The outstanding results of this analysis consists in the isolation of 0.37 per cent of glycine, 1.76 per cent of serine, and at least 10 per cent of hydroxyglutamic acid. These amino-acids have not been heretofore determined in the hydrolysis products of lactalbumin. A yield of 9.30 per cent of aspartic acid was obtained, which is nine times the amount previously recorded. These percentages, together with those of the other monoamino-acids, are tabulated and compared with the recorded results of a previous hydrolysis of lactalbumin.

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## THE ZINC AND COPPER CONTENT OF THE HUMAN BRAIN.

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(Received for publication, August 12, 1921.)

The presence of traces of copper in the human brain was first reported by Thudichum (1). In commenting on Thudichum's work Mathews (2) suggests that this point should be reinvestigated to see whether copper is in reality a normal constituent of all brains. He considers the possibility that the human brains which Thudichum examined might have come from brass-workers or others exposed to copper poisoning.

Despite the statement of Palet (3) who was unable to detect copper in 54 normal human livers there is general agreement among investigators that copper as well as zinc are normal constituents of plant and animal tissues. At the time the analyses recorded in this paper were begun no data concerning the occurrence of zinc in the human brain had appeared in the literature. Recently Rost (4) reported an analysis of a human brain containing 11 mg. of zinc per kilo.

Copper and zinc have been shown to be widely distributed in foods. The continuous ingestion of these metals raises the question as to the extent of their storage in various organs. The relative tolerance of zinc and copper by the animal organism, especially when introduced with foods has been noted by a number of observers. In a brief study of the fate of zinc in the animal organism, Salant, Rieger, and Treuthardt (5) found that after intravenous injection of zinc malate in cats the metal was stored in considerable amounts in the liver, that it was almost always found in the skin and muscles but that none was present in the brain. Giaya (6), on the other hand, finds that the partition of zinc per organ occurs in the following decreasing order: brain, lungs, stomach, liver, kidneys, intestines, heart, spleen.

*The Zinc and Copper Content of the Human Brain.\**

Case No.	Race or nationality.	Age.	Occupation.	Weight of brain. gm.	Amount taken for analysis.	Zinc.			Copper.			Cause of death.
						Found in sample.	In entire brain.	In 1,000 gm. of brain.	Found in sample.	In entire brain.	In 1,000 gm. of brain.	
				gm.	gm.	mg.	mg.	mg.	mg.	mg.	mg.	
1	Norwegian.	21	Seaman.	1,630	100	0.52	8.48	5.2	0.36	5.87	3.6	Fractured skull.
2	Negro.	23	Laborer.	1,157	100	0.88	10.18	8.8	0.60	6.91	6.0	Acute dilatation of the heart.
3	"	35	"		100	0.72		7.2	0.47		4.7	Angina pectoris.
4	Mexican.	40	"		100†	1.57		15.7	0.60		6.0	Lobar pneumonia.
5	White.	Fetus about 5 months.		64.5	64.5	0.74	0.74	11.5	0.44	0.44	6.8	Nothing to suggest disease of fetus.

\* The brains and autopsy records were kindly furnished us by Dr. H. C. Hartman and Dr. Anna M. Bowie of the Pathology Department.

† Analysis of the cerebrum. A separate analysis of the cerebellum of this brain gave 17.5 and 6.7 mg. per kilo of zinc and copper respectively.

Believing the subject to merit further attention we are presenting the results of the analyses of four adult brains and of a fetal brain. These were received in the laboratory immediately after autopsy, washed free of blood with physiological salt solution, and analyzed according to the methods previously described by Rose and Bodansky (7) and Bodansky (8, 9).

The results indicate that copper and zinc occur normally in the human brain, there being nothing in the records of any of the individuals to suggest exposure to zinc or copper poisoning. It will be observed that the values for copper fall within the limited range of 3.6 and 6.8 mg. per kilo. The proportion of zinc in the fetal brain was found to be greater than in three of the adult brains, the proportion of copper being greater than in any of the adult brains. A number of similar observations by other investigators may be mentioned. Ghigliotto (10) analyzed the viscera of a 7 months' old fetus and found the proportion of zinc to be slightly higher than in adults. Giaya (6) found 3 mg. of zinc in 100 gm. of a fetus weighing 420 gm. It appears that during intrauterine life there is more rapid accumulation of zinc and copper as well as of other inorganic constituents, than there is after birth. According to Fenger (11) the thyroids of beef fetuses contain more iodine and phosphorus per unit of body weight than thyroids from fully mature animals. The brain of a newly born albino rat contains greater proportions of phosphorus and sulfur than does the brain of an adult rat, according to the analyses of M. L. Koch (12). That there is a decrease in the ash content of the human brain with growth has been shown by W. Koch and Mann (13). In this connection it may also be of interest to recall that Maquenne and Demoussy (14) recently found in their studies on the migration of copper in the tissues of green plants that copper is most abundant in young actively growing tissues.

#### SUMMARY.

The results of the analyses of four adult brains and of a fetal brain indicate that copper and zinc are normal constituents of the human brain. Judging from our analysis of the one fetal brain, it appears that during intrauterine life there is more rapid storage of zinc and copper in the brain than there is after birth.

In this respect the behavior of these elements is similar to that of other inorganic constituents of animal tissues such as iodine, sulfur, and phosphorus.

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## A SIMPLIFIED FORM OF APPARATUS FOR AIR ANALYSIS.

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(Received for publication, July 22, 1921.)

The apparatus was designed primarily for general student use, therefore, simplicity and cost were considerations as well as reasonable accuracy. In service it has proved so satisfactory in general that it is described for the possible benefit of others. It differs from the well known forms in dimensions rather than in principles or in design.

It consists of a gas burette (Fig. 1, A) of bulb and stem form having a total calibrated capacity of 40 cc. From the tap to the upper calibration of the stem is 30 cc. The stem is calibrated from 30 to 40 cc. in 0.1 cc.

A water jacket of glass surrounds the bulb and stem of the burette (Fig. 1, B). It terminates below in a short neck. The internal diameter is about 8 mm. greater than the external diameter of the stem of the burette. The two stems are fixed and sealed together with a rubber connector at a point below the 40 cc. mark on the stem of the burette. The capacity of the jacket is about 90 cc. The totalunjacketed air space is about 1.5 cc. or 3 to 4 per cent. Three narrow cork wedges inserted between the outer wall of the bulb of the burette and inner surface of the expanded portion of the water jacket together with the rubber connector on the stem below firmly unites them. The stem of the jacket is securely attached to the base by two nicked spring clamps.

From the two-way tap, rise two short tubes bent at right angles. These tubes and all other glass tubes connecting the burette with the absorbers, including the stems of the latter, are thick walled and have narrow bores (about 2 mm.). The ends

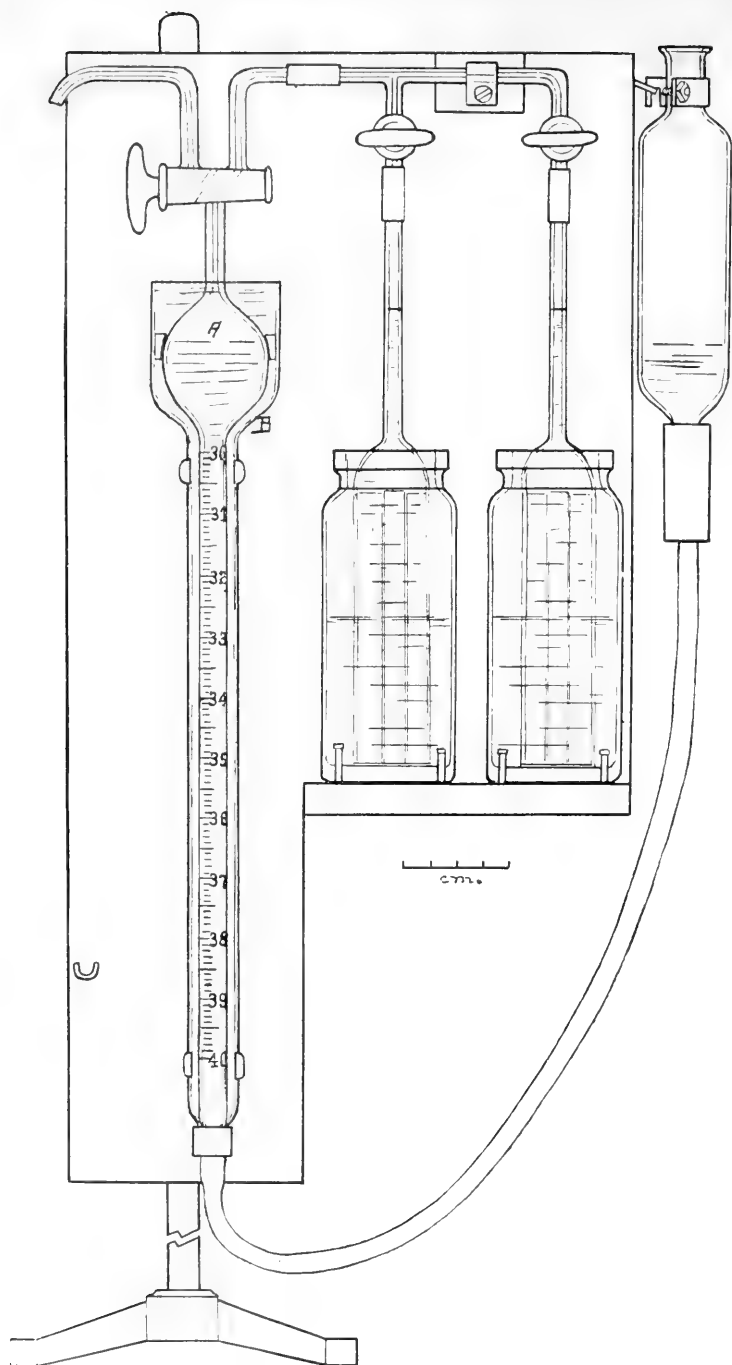


FIG. 1. Oxygen and carbon dioxide analyzer for expired air.



are slightly tapered to facilitate union by small bore, thick walled, pure rubber connectors. One of the bent tubes is for filling the burette and the other for connecting it with the tube leading to the absorbers. The free end of the filling tube is bent downward slightly in order that any displacing liquid forced through it will accumulate on the end, from which any surplus is conveniently removed by a sponge of absorbent material.

The tube leading to the absorbers consists of a horizontal part, a T and an inverted L limb. Near the middle of and integral with each of these limbs is a small one-way glass tap. The free horizontal end is attached to the burette by a short rubber connector. It is supported by a wood block, into a groove of which it is pressed by a nicked brass clamp fastened by a screw.

The absorbers are of tubular type and identical in construction. A horizontal mark or volume indicator is etched in the middle of the upper division or stem. The absorbers are attached to the T and L limbs by rubber connectors. The upper end of the absorber is dome-shaped and the stem rises from the center. The lower end is open and rests on the bottom of the absorber jacket which is an ordinary wide mouth 6 oz. bottle. The wall of the absorber is of ordinary thickness. The greatest external diameter is limited to 29 mm. which is slightly less than the internal diameter of the narrowest part of the bottle neck. To increase the absorbing surface, the absorber chamber is filled with a bundle of medium sized, thin walled, glass tubing, of such length that the upper ends extend to near the shoulders of the absorber when the lower ends are flush with the lower end of the absorber. When assembled and mounted, these tubes stand upon the bottom of the absorber bottles. Tubing of fair size is best for this purpose as it is efficient and gas bubbles do not stick among the tubes as may happen with slender tubing, particularly when very concentrated oxygen absorbing solutions are used. The absorber bottles are supported on a shelf which is provided with spring holders. These securely fix the bottles so that when assembled the apparatus can be moved about freely or transported. The holders are two slender brads driven perpendicularly part way into the shelf in such position that they press the bottles firmly against the front of the base behind the shelf.

The filling and displacing reservoir is an elongated, parallel sided glass bulb, having a capacity of 100 cc. The lower end terminates in a short stem, shaped for connecting with the rubber tube which leads to the gas burette. This tube has an external diameter of 8 mm. and wall of medium thickness. It is of pure rubber and is 45 cm. long. A thick rubber collar slipped over the connection with the stem of the reservoir provides a comfortable grip for handling it and guards against the temperature of the hand materially affecting the solution. The upper end of the reservoir is finished with a small neck with flaring mouth. To the neck is attached a metal hook for hanging the reservoir from staples driven part way into the base for the purpose. The hook is of wire, twisted about the neck and rendered immobile by winding with a strip of adhesive tape. A special nickeled hook with a clamp fastening with a screw is neater.

The base is 43.5 cm. long, 21.5 cm. wide, and 1.2 cm. thick. It is of soft wood, finished and painted black. Attached firmly to the back near the top and perpendicular to the surface, is a short nickeled rod for attaching by a clamp to a tripod stand. To the front, a wooden shelf is attached for supporting the absorbers and a wooden block for holding the glass tube connecting the burette and absorbers. The spring water jacket clamps are attached to the base with screws. The staples for suspending the displacing reservoir are driven part way into the base. One is near the top of each of the outer edges and another in the face near the left edge at about the level of the under surface of the absorber shelf.

#### *Solutions Employed.*

Distilled water containing 0.5 per cent (or less) of sulfuric acid and a little coloring matter (Orange G is satisfactory) is used in the reservoir for controlling the gas in the apparatus, about 75 cc. being a suitable amount. Owing to the comparatively short length of the gas burette, drainage is rapid, 3 minutes being the usual time allowed, even when measuring 40 cc. of gas. The pigment in the solution gives a visible index of drainage and also facilitates reading of the column of liquid on the graduation marks. A white strip of adhesive tape or paper pasted on the front of the support behind the stem of the burette further facilitates reading.

For absorbing carbon dioxide, 10 per cent aqueous sodium hydroxide solution is used, 100 cc. being introduced into the bottle of the absorber nearest the gas burette.

For absorbing oxygen an aqueous solution of potassium hydroxide and pyrogalllic acid, as recommended by Haldane<sup>1</sup> is used. Some samples of potassium hydroxide after the addition of the pyrogalllic acid, have given solutions of a grumous consistency in which case the addition of a little distilled water has rendered them satisfactory.

A layer (5 cc.) of paraffin oil is placed on top of the solution in the oxygen absorber to protect the solution from outside air. Analyzers set up with such solutions more than 6 months and used regularly and receiving no attention other than keeping the water jackets filled, show no deterioration in absorptive activity.

#### *Comments.*

For making atmospheric or expired air analyses, allowing 3 minutes for drainage yields good results. After reading the initial volume of air to be analyzed, it is passed five times into the carbon dioxide absorber, and after drainage, a new volume reading is taken. The air is then passed into the oxygen absorber ten times, then into the carbon dioxide absorber three times, to remove oxygen from the connections, and then back into the oxygen absorber ten more times and after drainage the final volume reading is taken. Of course a trace of oxygen will be left in the carbon dioxide absorber connections but if the oxygen absorber is in good condition the amount will be beyond the limits of the instrument to detect. If doubt exists, it is very easy to pass the air back into the carbon dioxide absorber again to control this point.

In displacing the air from the burette into the absorbers, care is taken to avoid entrance of the displacing liquid into the upper stem or tap of the burette.

The time required for carbon dioxide analysis, after the air is taken into the burette, is about 7 minutes and for a carbon dioxide and oxygen analysis, about 15 minutes. Perhaps it is trite to remark that such analyses should be undertaken only under suitable conditions, as uniform temperature of the liquids in the apparatus, room temperature, and absence of drafts.

<sup>1</sup> Haldane, J. S., *Methods of air analysis*, London, 2nd edition, 1918, 12, 13.

As Haldane points out the best practical control of such an instrument is the analysis of atmospheric air. With reasonable practice, consistent results, closely approximating the theoretical, are obtained for atmospheric oxygen.

TABLE I.  
*Results of Room Air Analyses.*

Date.	Carbon dioxide.	Oxygen.	Test No.	Operator.	Apparatus No.
1921	<i>per cent</i>	<i>per cent</i>			
Jan. 28	0	20.875	1	A	I
" 28	0	21.00	2	"	I
" 28	0	20.89	3	"	I
" 28	0	21.00	4	"	I
" 28	0	20.875	5	"	I
Mar. 9	0.05	20.95	1	B	II
" 9	0.05	20.95	2	"	II
" 9	0.05	20.95	3	"	II
" 9	0.05	20.925	1	"	I
" 11	0	20.95	1	A	VII
" 11	0.05	20.975	2	"	VII
" 11	0.05	20.975	3	"	VII

TABLE II.  
*Successive Analyses of Expired Air Taken from an 80 Liter Volume Held in a Bell Form of Spirometer.*

Time.	CO <sub>2</sub>	O <sub>2</sub>	Total.	Operator.	Apparatus No.
After 2 minutes.	4.00	16.43	20.43	B	I
" 7 "	4.00	16.45	20.45	A	VII
" 56 "	3.99	16.46	20.45	"	VII
" 62 "	4.02	16.33	20.35	B	I
" 302 "	3.98	16.43	20.41	A	VII
" 383 "	3.98	16.33	20.31	B	I

In view of its simplicity, compactness, portability, reliability, and speed of operation, the apparatus is well adapted for purposes permitting the limits of accuracy stated, as ordinary student or clinical use and certain types of experimental studies.

It permits of satisfactory performance of much work that otherwise would not be undertaken, owing to considerations such as the cost of standard forms, the time required to keep them in order

and to become proficient in their use and to conduct analyses. The glass parts were purchased at a cost of \$11.00. The burettes were calibrated and mounted in our shop.

### *Results.*

The results presented were selected to approximate average character and were obtained under ordinary conditions, so no doubt with greater refinement of technique, such as reading the scale with a lens, greater accuracy is possible. The skill of the operator, particularly in reading the burette is all important for minute accuracy as 0.01 cc. is estimated on the scale.

### SUMMARY.

A simplified form of analyzer for expired air is described which is well adapted for the use for which it was designed; *viz.*, general student, clinical, and certain types of experimental work.



## A GAS RECEIVER OF CONVENIENT AND PRACTICAL FORM FOR SAMPLING EXPIRED AIR FOR ANALYSIS.

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(Received for publication, July 22, 1921.)

In air analysis, perhaps the ideal technique is to take the sample directly into the gas burette, thus avoiding possibility of change in composition. Sometimes, however, it is desirable to collect and hold samples for subsequent analysis. The mercury receiver gives excellent results for collecting and holding samples, but for purposes where maximum accuracy is not essential, a less expensive and simpler method was desired. To meet this need, the method herein described, was developed.

A glass bottle having a capacity of 500 cc. and narrow, ground neck is employed as a receiver (Fig. 1). The bottle is provided with a two-holed rubber stopper, fitted with straight glass tubes of 5 mm. external diameter. The outer ends project about 1 cm. and over each of these is slipped the end of a 10 inch length of tight fitting, pure rubber tubing. Spring clamps are used for closing the rubber tubes. The inner end of one of the glass tubes terminates at the inner surface of the stopper, while the inner end of the other almost touches the bottom of the bottle when the stopper is firmly inserted into the neck. The holder consists of an ordinary rod stand about 65 cm. tall, fitted with two support rings, and a burette clamp adjusted to a single-hole stopper through which the end of an 8 cm. narrow stemmed glass funnel is thrust. The burette clamp is fastened near the top of the rod stand. The opening in the upper ring support is slightly larger than the widest diameter of the bottle and is fastened a little below the top clamp. The lower ring is less than the shoulder diameter of the bottle but greater than that of the neck and is placed about 5 to 10 cm. below the larger ring. The lower ring

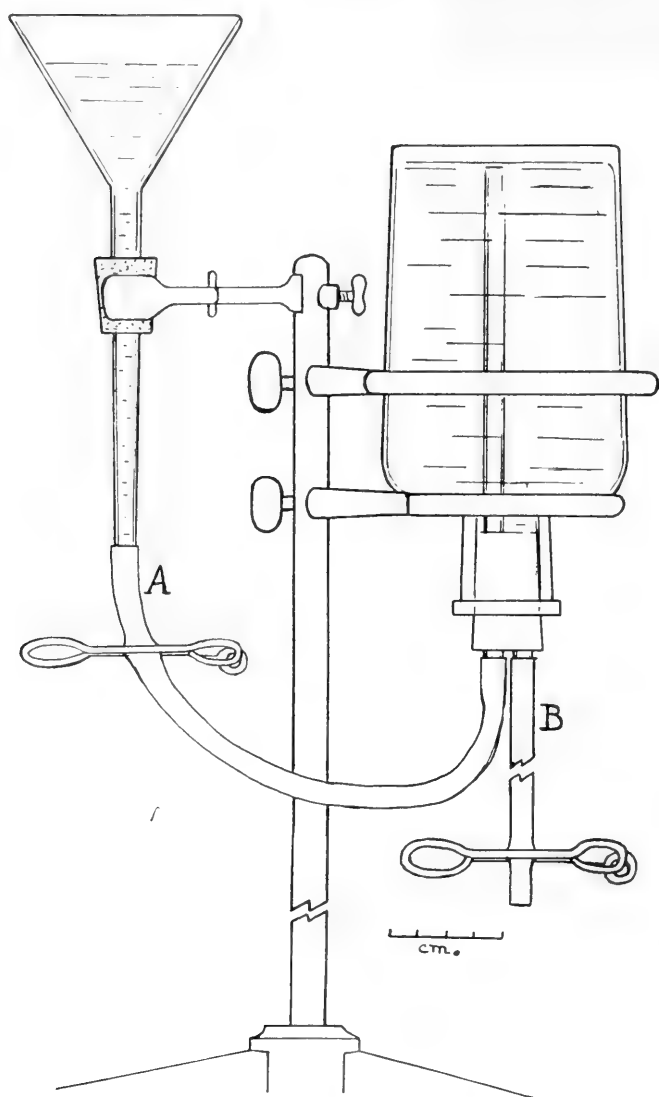


FIG. 1. Air sample receiver, Form 1. As shown it is filled with water, inverted, and is ready for disconnecting the funnel and filling with an air sample.



serves to support the weight of the bottle in either the upright or inverted position, the neck with stopper and rubber tubes extending through and below it in the latter position, while the upper ring maintains it perpendicularly and holds it on the lower one. Thus the upper ring serves as a guide while the lower one serves as a support or rest.

The bottle is filled by placing it in the upright position, attaching the stem of the funnel to the rubber tube connected with the glass tube that extends to near the bottom of the bottle, and pouring liquid into the funnel, the other rubber tube being open to permit the air in the bottle to escape. Water, to which a little phenol-sulfonephthalein has been added, is used for a filling and displacing liquid. When the bottle is almost filled, the funnel is disconnected and residual air from the lungs forced through the liquid in the bottle until the tint of the indicator becomes stabilized. The funnel is then reconnected and water from a flask, similarly saturated with expired air, is added until the bottle and tubes are full. The outer ends of the tubes are then clamped.

Using a funnel as described gives good results, the water used being saturated with expired air in a flask and poured into the funnel immediately before it is to run into the receiver. Conditions are favorable for loss of absorbed gas, however, owing to exposure to room air in pouring and also to the relatively large surface exposed in the funnel. Even so, considerable time of exposure in a funnel is required to produce a marked change in the carbon dioxide content and results show that but little error is introduced.

To reduce the objectionable features of the funnel and to render the technique more convenient and time-saving, a flask provided with a connection in the center of the bottom for the outflow tube is substituted for the funnel (Fig. 2). The flask is fitted with a stopper having three holes. Into one of these holes is fitted a short stemmed thistle tube, the lower end of which terminates just below the inner surface of the stopper. This is used for filling. One of the holes through the stopper is left open to provide a vent for the air in filling. Into the other hole is fitted a glass tube which extends to near the bottom of the flask. Its outer end is short and is bent to a right angle. To this is connected a rubber tube about 12 cm. long which serves as a connection for blowing expired air through the water in the flask, thus saturating

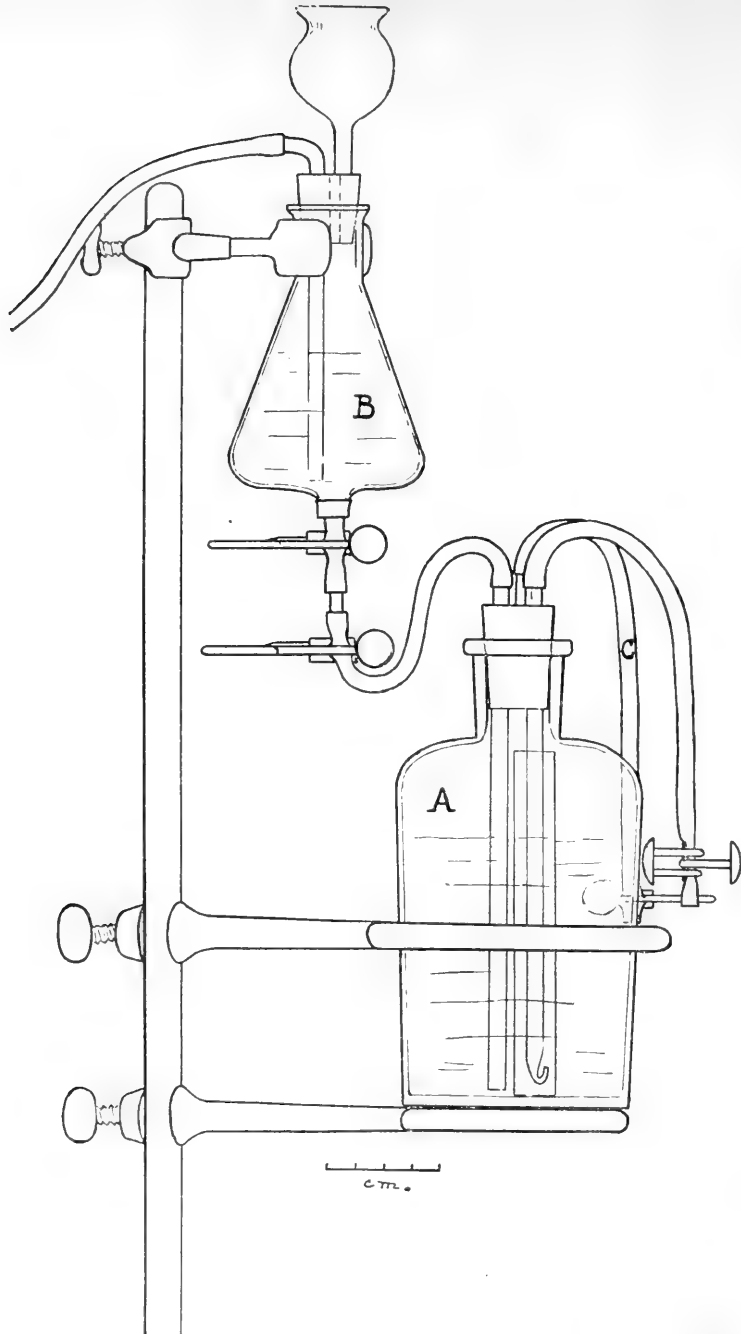


FIG. 2. Air sample receiver, Form II. Air sample receiver, A, with displacing reservoir, B. The tube C connects to the gas burette.<sup>1</sup>

<sup>1</sup> The receiver is a form designed for continuously sampling expired air in its passage through a mixing chamber. (For full description and method see article in the *Journal of Laboratory and Clinical Medicine*, now in press.)

and keeping it in this state. Owing to the almost closed condition of the flask, and the relatively small area of water exposed to the air as compared to the funnel, loss of gases from the water saturated in the flask type of reservoir is slower.

To collect a sample, the funnel or flask is disconnected and the bottle inverted (Fig. 1). The end of the filling tube is connected with the air reservoir to be sampled (avoiding, of course, any air in the connections other than air that is to be sampled), and the bottle filled by opening the rubber tube which acts as a siphon. As the water escapes, the air is drawn into the bottle, being delivered above the surface of the water by the long inside tube. When the bottle is filled with the air sample, both rubber tubes are clamped near the ends as before and disconnected from the spirometer.

When ready to analyze, the bottle is placed in the upright position and the rubber tube connected with the long glass tube, *i.e.* the filling tube, attached to the displacing reservoir (Fig 2). The reservoir is filled with water saturated with expired air from a flask that is kept stoppered excepting when removing its contents. Any air bubbles in the connections are removed by compressing the rubber tube or inserting a piece of wire. The clamp on the tube connected with the reservoir is then opened and water enters the bottle until the air is compressed and stops the flow. Care is exercised to keep the reservoir well filled to avoid the entrance of air. Water in the other rubber tube and connection is expelled by cautiously opening the clamp upon it with the tube in the dependent position. If care is not exercised in this operation, too much air is suddenly released, the reservoir empties too rapidly, and air enters the bottle.

The end of the tube is now ready for connection with the filling tube of the gas burette or analyzer. As the connection is made, a little air is permitted to escape from the receiver by slightly opening the clamp, in order that all other air in the connection may be displaced. As air passes into the analyzer, the pressure in the receiver falls and water enters from the reservoir.

#### *Comments.*

The loss of gas is accelerated after the introduction of water into the receiver. If a series of tests upon a single sample is desired the loss may be retarded by the introduction of a few cubic centi-

meters of paraffin oil into the receiver but this is objectionable owing to the action of the oil on the rubber parts.

Acidulating the water used in the receiver with sulfuric acid perhaps would be more satisfactory for this purpose.

### Results.

Results indicating the variations ordinarily encountered are shown in Table I.

TABLE I.

(a) Results of successive analyses of expired air taken directly into gas burettes from a spirometer containing 80 liters; (b) samples of the same air taken into receivers and allowed to stand for varying periods before analysis; (c) results from receivers showing the acceleration of loss of carbon dioxide after the introduction of water.

It should be noted that the figures for oxygen given in the table are not corrected for shrinkage of air volume, due to absorption of carbon dioxide. When thus corrected the actual change in oxygen content is very small.

	CO <sub>2</sub>	O <sub>2</sub>	Total.	Observer.	Instrument No.
Spirometer.					
After 7 minutes.....	4.00	16.45	20.45	A	VII
" 2 " .....	4.00	16.43	20.43	B	I
" 56 " .....	3.99	16.46	20.45	A	VII
" 62 " .....	4.02	16.33	20.35	B	I
" 302 " .....	3.98	16.43	20.41	A	VII
" 383 " .....	3.98	16.33	20.31	B	I
Receiver I.					
After 34 minutes.....	3.95	16.50	20.45	A	VII
" 38 " .....	3.95	16.38	20.33	B	I
" 74 " .....	3.95	16.50	20.45	A	VII
" 78 " .....	3.93	16.40	20.33	B	I
" 253 " .....	3.63	16.60	20.23	A	VII
" 253 " .....	3.65	16.67	20.32	B	I
Receiver II.					
After 275 minutes.....	3.98	16.47	20.45	A	VII
" 279 " .....	3.93	16.43	20.36	B	I
" 317 " .....	3.95	16.53	20.48	A	VII
" 302 " .....	3.98	16.40	20.38	B	I
" 322 " .....	3.93	16.43	20.36	"	I

### SUMMARY.

A simple method is described for collecting and storing samples of expired air for analyses which yields results having an error of about 1 per cent of carbon dioxide and considerably less than this for oxygen for periods of some hours.

## BACTERIA AS A SOURCE OF THE WATER-SOLUBLE B VITAMINE.

By SAMUEL R. DAMON.

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(Received for publication, August 10, 1921.)

In the literature dealing with the vitamine content of various substances there have been several allusions to the possible presence of vitamine in cultures of bacteria. Pacini and Russell (1) apparently demonstrated the presence of a growth-promoting substance in extracts of cultures of *Bacillus typhosus* which were fed to white rats that were limited to a diet devoid of water-soluble B. Thjötta (2) has shown that a favorable influence is exerted upon the growth of *Bacillus influenzae* by the addition of sterile bacterial extracts to the broth in which the organism is planted and he suggests that this may be due to the presence in the extracts of "substances belonging to the class of the so called vitamines." On the other hand Cooper (3) has found that extracts of *Bacillus coli* had no effect in relieving polyneuritis in pigeons.

It will be noted that in each of the cases cited above a different standard is employed by which the presence or absence of the vitamine is determined. The only factor that is common to all is the kind of vitamine that is under consideration, which in each case is the water-soluble, growth-promoting vitamine provided we assume the identity of the growth-promoting and antineuritic substances.

In this paper it will be shown that certain bacteria, at least, do not produce the growth-promoting substance known as water-soluble B. The organisms tested for this vitamine were *Bacillus paratyphosus* B, *Bacillus coli*, *Bacillus subtilis*, and whatever other organisms there are that make up the intestinal flora of the white rat when this animal is limited to a diet of known composition. The criterion employed to judge of the presence of vitamine was the weight curve of the growing white rat.

*Technique.*

An attempt was made to repeat the work of Pacini and Russell but all efforts to obtain a profuse growth of *Bacillus typhosus* on the same medium that they used, *i.e.* Uchinsky's medium, were unsuccessful. It was found possible, however, to get good growths of *Bacillus paratyphosus* B and *Bacillus coli*, and as these organisms all belong to the same general group they were substituted for *Bacillus typhosus* in this work. In the third experiment it was desired to add large quantities of one of the common forms of bacteria to the ration, so *Bacillus subtilis* was chosen and in this case plain nutrient agar was used as the culture medium.

The administration of the bacteria was carried out in the following way. The organisms were grown in flasks containing 100 cc. of the medium, then killed by autoclaving at 120° for 15 minutes—a procedure that would not effect the vitamine content as has been demonstrated by McCollum, Simmonds, and Pitz (4). The culture was then concentrated by evaporation on the steam bath to about 15 to 20 cc. and finally the organisms were taken up on starch by desiccation in a shallow pan at a reduced pressure. This starch bearing the bacteria was then used to replace an equivalent amount in the basal ration and the effect of bacteria noted by observing the trend of the weight curve.

The composition of the culture medium was:

	<i>gm.</i>
Asparagine.....	3.4
Ammonium lactate.....	10.0
Sodium chloride.....	5.0
Magnesium sulfate.....	0.2
Calcium chloride.....	0.1
Acid sodium phosphate.....	1.0

The basal ration used in all the experiments had the following composition:

	<i>gm.</i>
Casein.....	18.0
Starch.....	32.5
Sugar.....	17.0
Lard.....	15.0
Butter fat.....	10.0
Salt mixture.....	2.5

The salt mixture is that used by Osborne and Mendel (5) and has the following composition:

	gm.
Calcium phosphate.....	10.0
Acid potassium phosphate.....	37.0
Sodium chloride.....	20.0
Sodium citrate.....	15.0
Magnesium citrate.....	8.0
Iron citrate.....	2.0
Calcium lactate.....	8.0

The ingredients of the basal ration were mixed together in a mortar, the starch bearing the bacteria was added at this time, and the resulting paste was fed to the rats in small glass dishes.

By inspection it will be seen that the above ration is adequate in all dietary essentials with the exception of the water-soluble vitamine and it has been demonstrated repeatedly that it may be used to maintain young rats in good condition provided the deficiency of water-soluble B is not allowed to extend over too long a period.

#### EXPERIMENTAL.

*Experiment 1.*—Test of *B. paratyphosus* B for the water-soluble vitamine. In this experiment starch bearing the bacteria was used to replace an equivalent amount in the basal ration. The weight of wet bacteria added in this way to the diet was about 600 mg. per 100 gm. of ration. In Chart 1 is graphically represented the result of this test on two animals. It will be seen that the continued loss of weight of the animals was not prevented.

*Experiment 2.*—Test of *B. coli* for the water-soluble vitamine. The starch bearing the bacteria was added as in the above experiment and the weight of organisms was in this case approximately the same. Chart 2 shows the effect of this treatment on the weight curves of two of the rats. Again the loss of weight was not prevented.

*Experiment 3.*—Test of *B. subtilis* for vitamine. In this case the organisms were grown on nutrient agar, scraped off, weighed, and added to the basal ration. 20 gm. of bacteria were added to each 100 gm. of diet. It was realized that the nutrient agar itself contained the water-soluble vitamine and it was at first thought that this might be a source of error in the interpretation of the experiment. In Chart 3 are the weight curves of three rats limited to this diet. It will be noted that the loss of weight during the period before and after the addition of the bacteria to the diet is continuous.

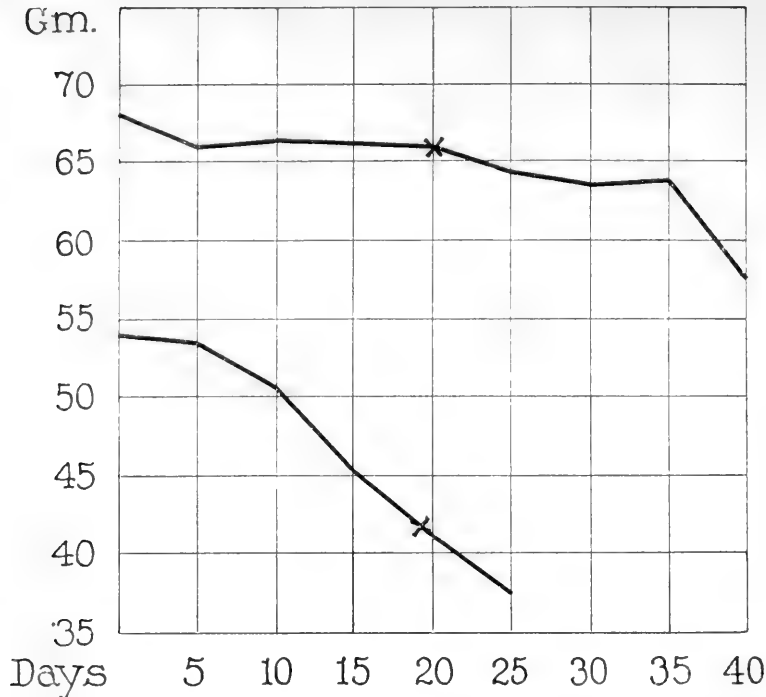


CHART 1. Up to the point marked (x) the rats were limited to a diet devoid of water-soluble B. At this point the bacteria were added to the diet. It will be seen that the loss of weight continues.

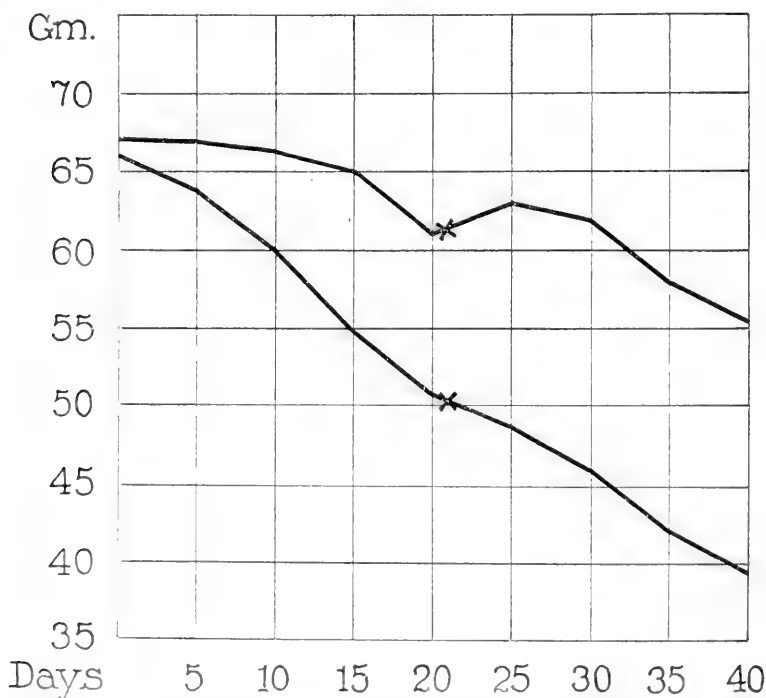


CHART 2. The preliminary diet was the same as in Experiment 1 but at the point indicated (x) *B. coli* were added to the ration. The loss of weight was not stopped.



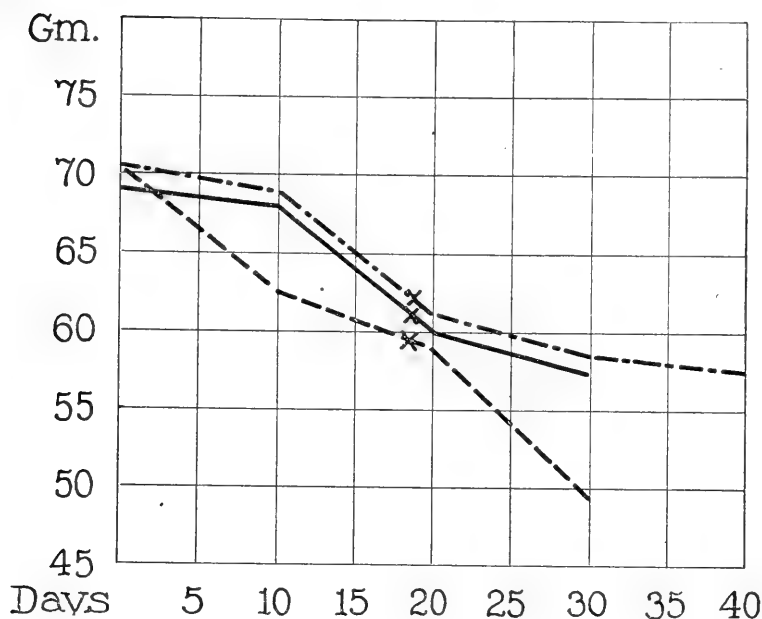


CHART 3. At the point marked (x) 20 gm. of *B. subtilis* were added to each 100 gm. of ration but the animals continued to lose weight.

#### DISCUSSION.

As already pointed out certain observers have apparently demonstrated the presence of a growth-promoting principle in bacteria. The standards by which they determined the presence or absence of this substance are not comparable however. So far as the author is aware the only way of measuring vitamine B that is not open to objection is by feeding the substance that is to be tested to rats that are being maintained on a diet that is devoid of this factor.

The experimental data presented above indicate that so far as *Bacillus paratyphosus* B, *Bacillus coli*, and *Bacillus subtilis*, are concerned there is no production of vitamine by these organisms. The objection may be raised that the quantity of bacteria added to the diet was not sufficient to affect the growth of the rats but it should be borne in mind that excessively small amounts of substances containing vitamine have been shown to produce a marked effect.

Regarding Thjötta's seeming demonstration of the production of a growth-promoting principle by mucoid bacilli and *Bacillus proteus* a further report will be made in a subsequent paper.

#### CONCLUSION.

*Bacillus paratyphosus* B, *Bacillus coli*, and *Bacillus subtilis* do not produce the growth-promoting principle known as water-soluble B vitamine.

The author desires to acknowledge the receipt of helpful suggestions from Professor F. P. Gorham.

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# THE CHARACTERISTICS OF CERTAIN PENTOSE-DESTROYING BACTERIA, ESPECIALLY AS CONCERNS THEIR ACTION ON ARABINOSE AND XYLOSE.\*

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PLATES 1 AND 2.

(Received for publication, August 2, 1921.)

Pentoses and pentose-yielding substances are found wherever the conditions are suitable for the growth of higher plants. A study of the changes these substances undergo indicates that there are present everywhere microorganisms which are able to bring about decomposition of the pentoses and related compounds. The almost universal distribution of the five-carbon compounds and the large quantities of plant products stored up in this form indicate the importance of studying the factors concerned in their decomposition.

The great family of organisms termed the lactic acid bacteria includes many forms which possess the power of splitting vigorously the pentose sugars. Perhaps the first recognition of the rôle of the lactic acid bacteria in the fermentation of pentoses was in 1894 when Kayser (1) isolated from sauerkraut an organism which fermented arabinose and xylose with the production of lactic acid. Since this time many reports of the fermentation of pentoses have appeared, Grimbert (2), Bertrand (3), and Bendix (4).

Gayon and Dubourg (5) studied the carbohydrate metabolism of the "mannit-forming bacteria" in considerable detail. Dubourg (6) reported additional studies of these organisms. Müller-Thurgau and Osterwalder (7, 8) greatly expanded the studies of Gayon and Dubourg, isolating and describing many new strains of pentose-fermenting bacteria of the general group called "mannit-forming bacteria." They also investigated the nature of the

\* This work was in part supported by a grant from the special research fund of the University of Wisconsin.

products obtained from this fermentation. Henneberg (9) described many types of pentose-fermenting bacteria of the lactic acid group. In general, the properties of the organisms are not described in sufficient detail to follow in the identification of unknown forms of lactic acid bacteria.

Orla-Jensen (10) in an exhaustive treatise on the lactic acid bacteria gives the results of a careful study of the protein metabolism, and the fermentation characters of 330 strains of lactic acid bacteria isolated from sour cabbage, beets, sliced potatoes, mash, and dough, and also from the excrement of cows, calves, and human beings.

We have shown in preceding papers (11, 12, 13) that a certain group of the lactic acid bacteria break down the pentose sugars with a high acid production, and the hexose sugars with a low acid and a high alcohol production. Since these first reports concerning the acid fermentation of xylose, our studies have been extended until at present we have results to show that there are other types of pentose-fermenting lactic acid bacteria commonly present in silage, sauerkraut, and related substances.

The present paper deals with the fermentative ability and general characteristics of a few members of the lactic acid family which destroy pentoses. It was the hope that such an investigation would give an insight into the physiology, the distribution, and the importance of these organisms in nature.

The cultures were obtained from various samples of corn silage and sauerkraut taken at different stages of their fermentation, usually between the 10th and 21st day. The structure of the colonies on the plates was studied and different types picked off into litmus milk and into measured amounts of 1 per cent xylose-yeast water and 1 per cent glucose-yeast water. From those showing acid production in xylose-yeast water, or a curd in litmus milk after 10 days, 12 were selected and replated. The selection of 12 cultures from the large number was based chiefly on the amount of acid formed from xylose, on the change noted in litmus milk, and on the source of the culture. It was planned to have representative types of the pentose fermenters which showed decided differences in degree of acid formation.

These 12 cultures fall into two groups; the organisms of Group I are readily distinguishable from those of Group II by their action

on milk and fructose. All of the strains of Group I coagulate milk slowly and do not form mannitol from fructose. Those of Group II form mannitol from fructose and do not coagulate milk. On the basis of fermentation reactions the bacteria of Group I may be divided into at least three strains, which differ with respect to the fermentation of certain carbohydrates. It is believed that the groups herein described are fairly representative of the rod forms of lactic acid bacteria that take part in the fermentation of pentose sugars.

### *Morphology of the Cultures.*

Although morphology is of little value in subdividing any group of organisms, it is possible by such a study to divide the lactic acid bacteria into coccus forms and short and long rods. All microscopical examinations were made from 24 hour glucose or xylose-yeast water agar slants or liquid-yeast water cultures incubated at 28°C.

Chinese ink preparations are especially useful in the study of morphology. It was noted that the organisms of the different groups and strains varied but slightly in size and shape. They are non-motile, usually blunt ended rods which occur singly or in twos, although long filaments or chains are sometimes noted, especially in liquid media. The different strains vary somewhat in size, especially in width of cells, from 0.5 to 0.8  $\mu$  wide and 1.25 to 3.00  $\mu$  long; Cultures 102, 31, and 32 are smaller, about 0.5 to 0.6  $\mu$  wide by 1.2 to 2.00  $\mu$  long. Even a single culture exhibits a wide variation in the size of the cells, hence morphology is of little varietal significance. Spores are not formed. The organisms stain easily with the ordinary aniline dyes and are Gram-positive. Photomicrographs of representative fields from microscopic preparations of the various groups are shown in Plates 1 and 2, Fig. 1, 2, 3, 4, and 5.

In accordance with the results of Beijerinck (14), these various groups of lactic acid bacteria were found catalase-negative; *i.e.*, without the ability to break down hydrogen peroxide.

The cultural characteristics of these organisms did not bring out any well defined differences. Briefly: They all grow best in a medium in which the source of nitrogen is yeast-water extract. Colonies are small and reach their maximum growth in

3 to 4 days. In stab cultures growth is moderate and uniform along the line of inoculation; on slants, scanty and beaded. Gelatin is not liquefied nor is casein digested. Growth in the acid range for both groups is stopped at a hydrogen ion concentration of about pH 3.5, while in the alkaline range for Group I it is stopped at about pH 9.0 to 9.4, and for Group II at pH 8.6 to 8.8. The optimum temperature for most of the organisms is about 30°C. and their thermal death-point is between 60 and 65°C.

The group, strain, and laboratory number, the source and the behavior in milk of these cultures are shown in Table I. For the sake of comparison, cultures of *Bacillus lactis acidi* and *Bacillus bulgaricus* are included. The value of milk in dividing these groups of bacteria is well illustrated in the figures of this table. Perhaps no other single physiological test so clearly defines the groups of lactic acid bacteria that ferment pentose sugars. As compared with *Bacillus lactis acidi* or *Bacillus bulgaricus*, none of these organisms forms large amounts of acid in milk; about 0.5 per cent of lactic acid is the maximum production after 2 weeks at 28°C. The time of curdling varies with the different cultures and with the temperature. At 38°C. curdling was more rapid than at 28°C. Group I, Cultures 29, 102-1, 124-1, 102, 31, and 32 require from 8 to 18 days, while Group II, Cultures 52, 52-7, 14, 57, and 118-8 fail to curdle milk. Unlike *Bacillus lactis acidi* the organisms of Group I did not reduce litmus until after curdling.

None of the lactobacilli of the pentose-fermenting group produced a firm curd so characteristic of the lactics commonly found in milk. On the contrary, the curd is soft and flocculent and it sinks leaving a  $\frac{3}{4}$  inch layer of whey on top.

*Sources of Nitrogen.*—All attempts to grow these various strains of lactic acid bacteria in peptone-phosphate medium (0.5 per cent each of dipotassium phosphate, peptone, and xylose), and in meat infusions resulted in a slow and scanty growth. Yeast water extract is far more suitable for growth and acid production than any other medium tested and for this reason it was made use of in all of these studies. To insure a medium low in fermentable carbohydrates and also low in organic acids, only fresh yeast was used and each batch of medium was carefully analyzed. A representative analysis was as follows: 0.15 cc. of N volatile acid, 0.75 cc. of N non-volatile acid, and 0.0508 gm. of nitrogen in 100 cc. of yeast water.

TABLE I.  
The Characteristics of the Pentose Fermenters in Milk.

Group.	Strain.	Culture No.	Source of cultures.	Milk at 28°C.				Effect on litmus.
				Time of curdling.	Kind of curd.	0.1 N acid.	Time of curdling.	
I	A	29 102-1	Sauerkraut.	46.8	14	Soft, whey on top. " " "	Reduced after curdling.	
			"	55.6	9		" "	
I	B	124-1 124-2	Sauerkraut.	46.0	9	Soft, whey on top. " " "	Reduced after curdling.	
			"	24.0	18		" "	
I	C	102	Sauerkraut.	18.8	18	Soft, whey on top. " " " " " "	Reduced after curdling.	
		31	Corn silage.	40.0	9		" " and	
		32	" "	37.2	9		white granules at top. Reduced after curdling and white granules at top.	
II		14	Corn silage.	No growth in milk. " " " " " " " " " " " "				
		118-8	Sheep manure.					
		57	Corn silage.					
		52	Sauerkraut.					
		52-7	"					
		<i>B. lactis acidi.</i>		82.8	2	Firm, without whey on top.	Reduced before curdling.	
		<i>B. bulgaricus.</i>		178.8	2	Firm, without whey on top.	" "	

## PART I.

*The Fermentation of Carbohydrates and Related Substances.*

The question of the constancy of the acid fermentation of sugars by bacteria has received much study. The value of this test, as well as its limitations, has been the object of so many careful investigations that no attempt is made in this report to review the literature. The results of various investigations (15-19) indicate that the power of an organism to form acid from carbohydrates or related substances is a characteristic not easily lost or acquired.

Although in this work no extensive study has been made of variations in fermentation reactions, it has been noted that cultures of *Lactobacillus pentoaceticus* which have been carried on glucose yeast-water agar for more than 2 years have not shown any well defined change of their fermentation reactions. The acid fermentation of sugars is without doubt the best possible means of differentiating members of the lactic acid group, provided a uniform and reliable method of determining fermenting power is used.

In a study of the acid production of these bacteria, 2 per cent solutions in yeast water of the carbohydrate or related substance to be fermented were employed. The 10 day cultures were titrated for total acidity, at which time a portion of the culture was removed for hydrogen ion determination and another portion for sugar analysis. The colorimetric method for the measurement of hydrogen ion concentration and the Shaffer and Hartmann (20) method of sugar analysis were used. To overcome as much as possible the decomposition of the sugar by sterilization, the more unstable compounds, arabinose and xylose, were sterilized in water solutions and added to the yeast water by means of sterilized pipettes. The xylose was prepared from corn cobs according to the method of Monroe (21) and recrystallized from alcohol until the correct specific rotation and freedom from heavy metals had been obtained. The other sugars, alcohols, etc., were Difco or Pfanstiehl preparations and were assumed to be true to label.

In these yeast water cultures, determinations of total acidity proved far more valuable as a measure of the degree of fermenta-



tion than determinations of the hydrogen ion concentration. Within a certain range, a change in the concentration of hydrogen ions is proportional to the acid formed, but does not give a true picture of the degree of utilization of the carbon compound. This criticism may also be directed towards the titration of total acidity but to a much less degree. These two acid determinations plus the sugar analysis give a fairly accurate evaluation of the nature of the fermentation. Support for these statements will be found in the tables that follow.

*The Fermentation of Arabinose, Xylose, and Rhamnose.*

In Table II are assembled the analyses for acid production and sugar consumption from the breaking down of arabinose, xylose, and in the case of rhamnose, acid production alone. Where the acidity is below 0.5 to 0.6 per cent of normal acid it was assumed that there was no fermentation. A small amount of acid may be due to the action of the bacteria on the substance present in yeast water and to a slight decomposition of the carbohydrate during sterilization. In every case the figures given in this and in other tables are the result of subtracting this acid from that in the fermented culture.

All of the bacteria described in this paper ferment arabinose with the production of considerable amounts of acid. The various strains show a well defined difference in their ability to form acid; 10 days after inoculation cultures of the organisms of Strain A contain about 5.0 per cent, Strain C about 8.9 per cent, and Strain B about 11.3 per cent of normal acid, Table II. The high acid production which is characteristic of Strain B is also noted in the case of all of the organisms of Group II. A point of special interest shown in the results presented in this table is the close agreement between sugar fermented and acid formed. Perhaps in no other table is this correlation so evident. These results indicate that the chief products of the fermentation are acids rather than neutral or highly volatile bodies. *Bacillus lactis acidi* did not ferment arabinose, xylose, or rhamnose. On the basis of total acid formed from xylose, the bacteria of Group I may be arranged in three divisions: Strain A and B, low acid, and Strain C, no acid, or only a trace. All organisms of Group II are high acid formers.

TABLE II.  
*The Fermentation of Pentoses.*

Group.	Strain.	Culture No.	Calculated for 100 cc. of culture.									
			Arabinose.			Xylose.			Rhamnose.			
			0.1 N acid.	pH	Sugar fermented.	0.1 N acid.	pH	Sugar fermented.	0.1 N acid.	pH		
			cc.		gm.	cc.		gm.	cc.			
I	A	29	53.1	4.0	0.410	47.5	3.8	0.388	37.0	5.6		
		102-1	46.5	3.8	0.257	39.9	3.8	0.187	35.6	4.0		
I	B	124-1	114.0	3.2	0.950	58.4	3.8	0.375	21.6	4.2		
		124-2	113.0	3.2	0.950	58.9	3.8	0.355	25.4	4.0		
I	C	102	86.0	3.6	0.554	6.1	4.6	0.014	7.5	5.4		
		31	91.5	3.4	0.530	7.0	4.8	0.018	34.8	3.8		
		32	89.0	3.4	0.596	7.5	4.8	0.045	36.4	3.8		
II		14	122.4	3.2	0.950	105.3	3.2	0.874	6.5	5.6		
		118-8	124.3	3.2	0.950	94.7	3.2	0.874	5.4	6.0		
		57	118.5	3.4	0.950	102.0	3.4	0.874	6.8	5.6		
		52	103.8	3.4	0.950	104.2	3.4	0.874	7.2	5.4		
		52-7	111.0	3.2	0.950	104.1	3.2	0.874	1.4	6.4		
<i>B. lactis acidi.</i>			5.7	4.4		5.9	4.8		7.8	5.4		

Cultures 29, 102-1, 124-1, and 124-2 of Group I formed from xylose only about half as much acid as the cultures of Group II and likewise consumed much less sugar. The fermentation of xylose will be discussed more fully in a later table. Repeated tests show that Cultures 102, 31, and 32 do not ferment xylose, or if so, only to a slight degree.

Rhamnose is fermented by all of the cultures of Group I but not by any of the cultures of Group II. As compared with the other sugars the amount of acid produced from rhamnose is small.

The significant fact brought out in the figures of this table is the marked difference in fermentation of the two sugars, arabinose and xylose. In 10 days, Cultures 124-1 and 124-2 destroy 1 per cent of arabinose and form more than 11 per cent of normal acid, while on xylose less than half of this amount of sugar is consumed and 5.8 per cent of normal acid formed. Strain C, represented by Cultures 102, 31, and 32, readily attacks arabinose but not xylose.

*Progressive Fermentation of Xylose.*—The high acid from arabinose and the low acid from xylose formed by Cultures 124-1 and 124-2 cannot be explained on the ground that these organisms are sensitive to an acid reaction, for with arabinose the total acid and the true acidity are far greater than with xylose. It is possible that this strain attacks xylose much more slowly than arabinose. To test this point an experiment was made in which the formation of acid from xylose was measured at different times. Titrations after 3, 8, 10, and 30 days were made. No decided increase in acid formation was noted in the cultures kept for 30 days. Apparently the total acid after 10 days is not greatly increased by longer incubation.

It was noted that the rate of acid production is rapid, within 3 days approximately one-half of the total acid formed from xylose is obtained.

#### *The Fermentation of Glucose, Galactose, Mannose, and Fructose.*

The complete results of the fermentation of these sugars are given in Table III. The chief point of interest in the table is the high acid production by the bacteria of Group I and the low acid production by the bacteria of Group II. While the total acid

TABLE III.  
*Fermentation of Hexoses.*

Culture No.	Calculated for 100 cc. of culture.											
	Glucose.			Galactose.			Mannose.			Fructose.		
	0.1 N acid.	pH	Sugar fer- mented.	0.1 N acid.	pH	Sugar fer- mented.	0.1 N acid.	pH	Sugar fer- mented.	0.1 N acid.	pH	Sugar fer- mented.
	cc.		gm.	cc.		gm.	cc.		gm.	cc.		gm.
29	123	3.4	0.880	101.6	3.4	0.863	122.4	3.4	0.974	101.7	3.4	0.609
102-1	122	3.4		104.0	3.6		112.0	3.6		93.6	3.6	
124-1	115	3.4	0.860	92.6	3.6		122.4	3.4		95.9	3.6	
124-2	130	3.4		93.4	3.4	0.786	135.6	3.4	1.11	105.6	3.4	0.944
102	95	3.4	0.940	81.0	3.6	0.667	102.0	3.4		97.0	3.6	0.828
31	111	3.4		100.9	3.4		114.4	3.4	0.848	101.8	3.4	
32	102	3.4		94.7	3.4		107.6	3.4		100.3	3.6	
14	78	3.6	1.090	49.8	3.8	0.798	30.2	4.2	0.714	65.4	3.8	
118-8	70	3.6		52.6	3.6		18.6	4.4		63.8	3.8	
57	64	3.6		50.9	3.8		15.2	4.4		61.0	3.8	
52	65	3.6		53.8	3.6		18.6	4.4		62.8	3.8	
52-7	71	3.5	1.090	53.7	3.6	0.833	19.6	4.4	0.720	67.6	3.8	1.473
<i>B. lactis acidi.</i>	125	3.4		103.9	3.6		117.8	3.4		110.8	3.6	

formed varies somewhat with the different organisms, there is no significant difference in acid production from the various sugars. The results of previous work with Culture 118-8 of Group II, have shown that glucose and other aldo-hexoses are broken down with the formation of a neutral substance; *i.e.*, ethyl alcohol. As much as 25 per cent of the sugar may be accounted for as ethyl alcohol. This type of fermentation is noted with glucose, galactose, and mannose, while with fructose the polyhydric alcohol mannitol is found in place of ethyl alcohol. Because of their power of forming mannitol many investigators (5, 7, 8, 22) have given to these organisms the general name of "mannit-forming bacteria." As shown in Table VI all the cultures of Group II ferment mannitol. Here then is a group of bacteria which forms from fructose a product, mannitol, and then ferments this product. The fermentation of fructose by Culture 118-8 has been studied quantitatively and it was shown that about 40 per cent of the fructose is converted into mannitol. The products resulting from the fermentation of the mannitol are acetic acid, lactic acid, and  $\text{CO}_2$ . Two minor points brought out by the results of this fermentation are the low acid formed from galactose by the bacteria of Groups I and II, and the low acid from mannose by the bacteria of Group II.

As will be shown in a later table the bacteria of Group II agree in general with the observation of Orla-Jensen (10); namely, that little or no fermentation of mannose is usually indicative of a similar behavior towards salicin.

#### *The Fermentation of Sucrose, Maltose, and Lactose.*

The wide range of fermentative reactions of the lactic acid bacteria is shown from the figures of Table IV. All of the strains of Group I form acid in the breaking down of sucrose, maltose, and lactose. Cultures 29 and 102-1 form approximately 10.0 per cent of normal acid from sucrose, maltose, and lactose. On the other hand Cultures 124-1 and 124-2 produce about 12.0 per cent of normal acid from sucrose, about 10.5 per cent from maltose, and about 8.7 per cent from lactose. The three cultures belonging to Strain C exhibit decided differences in their fermentation of these sugars. Regardless of the sugar, Culture 102 produced the lowest

total acid of any of the organisms included in Group I. Cultures 31 and 32 break down sucrose with a high acidity, about 10.7 per cent of normal acid, maltose with a much lower total acidity about 8.7 per cent, and lactose with a high total acidity of about 10.8 per cent.

In the fermentation of these three disaccharides, the organisms of Group II behaved very differently from those of Group I. In

TABLE IV.  
*Fermentation of Disaccharides.*

Culture No.	Calculated for 100 cc. of culture.								
	Sucrose.			Maltose.			Lactose.		
	0.1 N acid.	pH	Sugar fermented.	0.1 N acid.	pH	Sugar fermented.	0.1 N acid.	pH	Sugar fermented.
	cc.		gm.	cc.		gm.	cc.		gm.
29	94.0	3.4		95.0	3.4	0.955	102.7	3.4	0.930
102-1	101.0	3.4		107.5	3.2		92.0	3.4	
124-1	118.0	3.4		103.8	3.4		87.5	3.4	1.060
124-2	120.0	3.4	0.946	107.3	3.4	1.070			
102	86.0	3.4	0.583	78.5	3.4	0.960	71.0	3.6	0.670
31	108.0	3.4		87.0	3.6		111.0	3.3	
32	106.0	3.4		87.3	3.4		106.5	3.4	
14	54.5	3.6	0.500	61.5	3.6	1.150	23.0	4.4	0.380
118-8	42.2	3.6	0.205	61.0	3.6	1.118	8.0	5.6	
57	41.1	3.6		56.3	3.6		6.0	5.8	
52	19.9	4.6		65.0	3.6	1.070	6.0	5.6	
52-7	10.1	5.6		60.0	3.6		7.0	5.6	
<i>B. lactis acidi.</i>				61.6	3.4		102.0	3.4	

general these mannitol-forming bacteria produced about 5.6 to 6.5 per cent of normal acid from maltose, a somewhat lower percentage of acid from sucrose, and only a trace of acid from lactose. Culture 14 offers an exception, it attacks lactose. Although not all the cultures of Group II break down sucrose and maltose with the same final yield of acid, the differences are not great enough to be of use in dividing this group. Apparently Culture 14 possesses a slightly different fermentative ability from any of the

other members of this group. If acid production of these various groups of bacteria be compared with that of *Bacillus lactis acidi* it will be seen that there is a decided difference; the latter attacks maltose slowly, and lactose readily.

From the sugar determinations, it appears that in certain cases the sugar consumed is not proportional to the acid formed. Perhaps neutral substances are formed during the fermentation. For an example, see the figures obtained from the fermentation of maltose by the bacteria of Group II.

*Progressive Fermentation of Lactose.*—The rate of the fermentation of lactose was measured after 4, 7, and 10 days. It was found that the fermentation proceeds so rapidly that within 4 days after inoculation the greater part of the acid is formed. From the 4th to the 10th day the acid content increases but slowly. If calculated as lactic acid the total sugar fermented is approximately equal to the acid formed. One point of special interest noted in the results of this fermentation is the variation in acid production of the organisms of Strain C. It was noted that the total acid formed by Culture 102 from lactose is much less than that formed by Cultures 31 and 32.

*The Fermentation of Melezitose, Raffinose,  $\alpha$ -Methyl Glucoside, Salicin, and Esculin.*

The results of the fermentation of the trisaccharides, melezitose and raffinose, and the glucosides,  $\alpha$ -methyl glucoside, salicin, and esculin, are given in Table V. The trisaccharide, melezitose, is not easily decomposed. Because of the decided difference in the availability to these organisms as measured by the production of acid, melezitose is an important sugar in the differentiation of these bacteria. The organisms of Strain C attack this trisaccharide vigorously, producing about 10 to 11 per cent of normal acid from a 1 per cent solution of the sugar. It is significant that with the exception of a very small acid production by the organisms of Strain B none of the other bacteria attacked melezitose.

Raffinose is far more available for the lactic acid bacteria than melezitose. Except in the case of the organisms of Group II and *Bacillus lactis acidi*, raffinose is decomposed with the production of large amounts of acid. It does not furnish any characteristic

fermentation reactions for the pentose-destroying bacteria of Strains A, B, and C.

$\alpha$ -Methyl glucoside is readily fermented by the majority of the organisms of Groups I and II. The various strains of Group I exhibit well defined differences in their power of acid production from this glucoside; Strain B forms large amounts of acid, Strain A approximately half as much acid as Strain B, and Strain C does

TABLE V.  
*Fermentation of Trisaccharides and Glucosides.*

Calculated for 100 cc. of culture.									
Culture No.	Melezitose.		Raffinose.		$\alpha$ -Methyl glucoside.	Salicin.		Esculin.	
	0.1 N acid.	pH	0.1 N acid.	pH	0.1 N acid.	0.1 N acid.	pH	0.1 N acid.	pH
	cc.		cc.		cc.	cc.		cc.	
29	9.4	5.6	91.6	3.4	21.8	81.6	3.4	36.3	4.4
102-1	7.6	5.2	83.6	3.5	23.1	91.2	3.4	36.3	4.2
124-1	10.0	5.2	82.8	3.6	48.0	80.6	3.6	38.2	4.2
124-2	11.6	5.2	83.0	3.6	60.5	80.0	3.6	38.2	4.0
102	106.4	3.6	83.6	3.6	7.6	90.6	3.6	34.8	4.0
31	110.4	3.4	81.6	3.6	7.6	56.2	3.6	36.4	4.0
32	112.6	3.2	83.8	3.4	7.1	78.0	3.6	33.5	4.4
14	6.8	5.6	2.2	6.0	43.8	12.8	5.0		7.2
118-8	7.6	5.6	5.0	5.8	47.0	5.0	6.2		7.2
57	6.4	5.8	5.0	5.8	41.8	4.0	6.2		7.2
52	8.0	5.4	5.2	5.6	45.2	3.2	5.6		7.2
52-7	5.6	6.2	6.4	5.6	46.2	2.4	6.4		7.2
<i>B. lactis acidi.</i>	9.6	5.1	6.2	5.6	16.7	21.6	4.2		7.2

not attack  $\alpha$ -methyl glucoside. Because of the decided difference in the fermentation of  $\alpha$ -methyl glucoside by the lactic acid bacteria isolated from fermenting plant tissue, it is important in subdividing the various strains of these organisms. It is not attacked by the bacteria of Strain C. All the organisms of Group II form acid from  $\alpha$ -methyl glucoside.

The two glucosides, salicin and esculin, are decomposed by the organisms of Strains A, B, and C, but are resistant to the organ-



isms of Group II. An exception to this statement is seen in the case of Culture 14 where a slight fermentation is noted. In every case the total acid formed from esculin is far less than that from salicin.

*Fermentation of Mannitol, Glycerol, and Dulcitol.*

The polyhydric alcohols, mannitol, glycerol, and dulcitol furnish sources of carbon for the separation of the lactic acid bac-

TABLE VI.  
*Fermentation of Polyhydric Alcohols.*

Culture No.	Calculated for 100 cc. of culture.					
	Mannitol.		Glycerol.		Dulcitol.	
	0.1 N acid.	pH	0.1 N acid.	pH	0.1 N acid.	pH
	cc.		cc.		cc.	
29	42.0	3.5	36.0	3.8	6.2	5.4
102-1	56.0	3.5	33.2	3.9	6.0	5.4
124-1	49.0	3.6	25.6	4.0	39.8	3.8
124-2	58.0	3.7	27.0	4.0	36.4	3.8
102	46.0	3.6	17.6	4.4	6.0	5.4
31	57.0	3.6	14.6	4.0	4.6	5.8
32	55.0	3.6	13.8	4.2	5.6	5.6
14	20.0	4.4	8.6	5.8	4.0	6.6
118-8	15.2	4.3	5.4	6.0	3.6	6.2
57	15.6	4.4	5.0	6.2	3.4	6.6
52	16.2	4.2	4.8	6.0	3.8	6.6
52-7	19.0	4.2	3.8	6.0	3.6	6.4
<i>B. lactis acidi.</i>	46.0	3.8			7.4	5.6

teria into groups, as shown in Table VI. Of the three alcohols dulcitol is by far the most important for a study of fermentation reactions. It is not attacked by any of the lactic acid organisms used in this study except Cultures 124-1 and 124-2. In this respect our results agree with those of Orla-Jensen (10) who says that lactic acid bacteria which ferment dulcitol are extremely rare. According to Winslow and his associates (23) dulcitol occupies a unique position in the fermentation test in that it does not corre-

late with the other carbohydrates. From our results it appears that the fermentation of dulcitol is highly specific and may be used to separate closely related strains of lactic acid bacteria. Mannitol is fermented by all of the organisms of both groups, although much more slowly by those of Group II. Glycerol is fermented even more slowly than mannitol and very slightly by the organisms of Group II.

In addition to the substances already described fermentation tests were carried out with starch, dextrin, and inulin, but no appreciable acid production was noted.

#### *Distinctive Fermentation Characteristics.*

The fermentation of certain carbohydrates and related compounds furnishes a means of separating into well defined groups the lactic acid bacteria that ferment the pentose sugars. Arabinose, xylose,  $\alpha$ -methyl glucoside, melezitose, and dulcitol have been found especially useful in the separation of the different strains of Group I. 1 per cent xylose-yeast water is easily the most valuable medium in the separation of these lactic organisms into different groups. According to the amounts of acid formed these organisms naturally fall into three, and possibly four, divisions. The separation of Strains A and B, is based solely on the variation in the amount of acid produced, approximately 4.4 per cent of normal acid for Strain A, and 5.8 per cent for Strain B. It is only fair to say that the fermentation of xylose does not furnish a clear-cut separation of the organisms of these two groups. The production of acid from dulcitol is an easily distinguishable characteristic of the organisms of Strain B, while those of Strain A will not attack this polyhydric alcohol. The bacteria of Strain C are readily separated from the other organisms of both groups by the fermentation of melezitose.

The principal results of the fermentation tests are grouped in Table VII. The figures of this table bring out clearly the most interesting facts obtained in this preliminary study of the pentose-fermenting lactic acid bacteria. Here the fermentation of the test substance is indicated by numbers. The data are arranged to bring out the degree of fermentation. For example, in the case of Culture 29 the figure 3 for arabinose and xylose indi-

cates a fair acid production while 6 for hexoses and disaccharides indicates a strong fermentation. The figures of this table show the approximate amount of acid formed by the several organisms from the breaking down of the various carbohydrates and related compounds.

TABLE VII.  
*General Fermentation Characters of the Pentose Bacteria.*

Carbon compound.	Group I.							Group II.				
	A		B		C							
	29	102-1	124-1	124-2	102	31	32	14	118-8	57	52	52-7
1. Arabinose.....	3*	3*	6*	6*	5*	6*	5*	6	6	6	6	6
2. Xylose.....	3*	2*	3*	3*	0*	0*	0*	6	6	6	6	6
3. Rhamnose.....	2	2	1	1	0	2	2	0	0	0	0	0
4. Glucose.....	6	6	6	6	6	6	6	5	4	4	4	5
5. Galactose.....	6	6	6	6	5	6	6	3	3	3	3	3
6. Mannose.....	6	6	6	6	6	6	6	2	1	1	1	1
7. Fructose.....	6	6	6	6	6	6	6	4	4	4	4	4
8. Sucrose.....	6	6	6	6	5	6	6	3	2	2	1	0
9. Maltose.....	6	6	6	6	5	5	5	4	4	3	4	4
10. Lactose.....	6	6	5	—	4	6	6	1	0	0	0	0
11. Melezitose.....	0*	0*	0*	0*	6*	6*	6*	0	0	0	0	0
12. Raffinose.....	6	5	5	5	5	5	5	0	0	0	0	0
13. $\alpha$ -Methyl glucoside.....	1*	1*	3*	4*	0*	0*	0*	2	3	2	2	3
14. Salicin.....	5	5	5	5	6	3	5	0	0	0	0	0
15. Esculin.....	2	2	2	2	2	2	2	0	0	0	0	0
16. Mannitol.....	2	3	3	3	3	3	3	1	1	1	1	1
17. Glycerol.....	2	2	1	1	0	0	0	0	0	0	0	0
18. Dulcitol.....	0*	0*	2*	2*	0*	0*	0*	0	0	0	0	0

\* These figures represent the fermentation tests especially useful in separating the different strains of Group I.

The arbitrary standards adopted were as follows:

0 equals	0-15 cc. of 0.1 N acid.
1	" 15-30 " "0.1 " "
2	" 30-45 " "0.1 " "
3	" 45-60 " "0.1 " "
4	" 60-75 " "0.1 " "
5	" 75-90 " "0.1 " "
6	" 90 or above cc. of 0.1 N acid.

Among the compounds most valuable for differentiation are lactose, fructose, melezitose, the pentoses, and the higher alcohol, dulcitol. This separation is not dependent upon acid formation alone but also on the production of neutral bodies, for example, mannitol from fructose. It is realized that the grouping adopted in this paper may bring together bacteria related in only one character but not in other characters; however, it is the best means at hand to separate the great complex of lactic acid bacteria.

## PART II.

### *Quantitative Determination and Identification of the Products Formed from Arabinose and Xylose.*

Arabinose and xylose are easily destroyed when sterilized in a slightly alkaline solution, and undergo a small amount of decomposition even when sterilized in yeast water. To avoid this change an 8 per cent water solution of each pentose was sterilized and then by means of a sterilized pipette added to the yeast water until the concentration was about 2 per cent. The exact strength was determined by analysis, which usually gave from 1.91 to 2.04 per cent of the pentose. The fermentation flask was equipped with a carbon dioxide absorption bottle similar to that described in a previous paper (12). Sterilized brom-cresol purple was added to the fermenting solution at the time of inoculation to indicate the formation of acids. Whenever a strong acid reaction was apparent, sterilized  $\times$  sodium hydroxide was added until the solutions were approximately neutral. In this way, it was possible to measure the rate of fermentation and also to determine when fermentation had ceased. Occasionally fresh additions of brom-cresol purple were necessary as the indicator is partly destroyed in the fermentation process. When acid formation ceased, usually after 10 to 14 days, the cultures were analyzed for carbon dioxide, unfermented sugar, volatile and non-volatile acids, and alcohol.

*Methods of Analysis.*—Carbon dioxide, volatile and non-volatile acids, and alcohol were determined as described in previous publications (11, 12). The distillate was analyzed by the method of von Fürth and Charnass (24) for lactic acid carried over with

the volatile acid, but in no case were more than a few milligrams found. A correction corresponding to the quantity found has been applied to the values for acetic and lactic acids.

Sugars were determined by the titration method of Shaffer and Hartmann (20). This is a rapid volumetric method that gives practically the same accuracy as the longer gravimetric methods. The method was tested with purified xylose and arabinose and found to give quantitative recovery of the sugars both from water and from culture solutions.

Analyses were made of the uninoculated medium for volatile and non-volatile acids, blanks were run on the reagents, and the values thus obtained were subtracted from the corresponding determinations of the fermented cultures.

In the regular procedure 400 cc. of an approximately 2 per cent sugar solution were fermented. Due to the addition of  $N$  NaOH to neutralize the acids formed, the volume at the end of the fermentation was in the neighborhood of 475 cc. Of this, 200 cc. were used for the determination of volatile and non-volatile acids, 100 cc. for alcohol, 50 cc. for sugar, 5 to 10 cc. for carbon dioxide, and the remainder kept in the ice box as a reserve in case a determination should be lost.

The fermentation of the pentoses was rapid and usually complete in about 14 days. Of the two, arabinose was more rapidly fermented than xylose. Very little sugar remained unfermented, rarely more than 0.1 gm. and sometimes as little as 0.05 gm. to 100 cc. of culture. Of the fermented sugar about 95 per cent is accounted for by the products. The volatile and non-volatile acids, which later will be shown to be acetic and lactic acid respectively, comprise 98 per cent or more of the total products. No measurable quantity of alcohol was found and the carbon dioxide was never more than 0.046 gm. for 100 cc. of culture. The acetic acid and lactic acid are produced at approximately the ratio of 1 molecule of acetic for 1 molecule of lactic. The ratio of their molecular weights is 1:1.50 and the ratios found vary from 1:1.34 to 1:1.52. Somewhat lower ratios were obtained from the mannitol-forming group, the bacteria of which have the power of slowly fermenting lactic acid to acetic acid and carbon dioxide. When all, or nearly all, of the sugar has been fermented, it is probable that the bacteria attack the lactic acid formed and

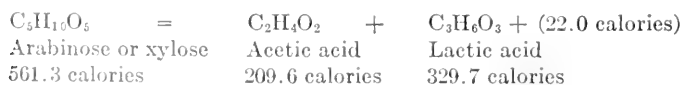
so increase the proportion of acetic acid at the expense of the lactic acid. The absence of any appreciable amount of arabinose or xylose (less than 0.05 gm. to 100 cc. of culture) and a rather high production of carbon dioxide lends support to this view. The data are given in Table VIII.

TABLE VIII.  
*Total Fermentation Products from Arabinose and Xylose.*

Culture No.	Sugar.	Calculated for 100 cc. of culture.					
		Weight of sugar fermented.	Acetic acid.	Lactic acid.	Ratio of acetic to lactic.	Carbon dioxide.	Sugar accounted for by products.
		gm.	gm.	gm.		gm.	per cent
29	Arabinose.	1.73	0.653	0.992	1:1.52	0.023	96
29	Xylose.	1.79	0.695	1.022	1:1.48	0.023	97
124-2	Arabinose.	1.81	0.688	1.004	1:1.46	0.017	90
124-2	Xylose.	1.73	0.644	0.936	1:1.45	0.024	93
102	Arabinose.	1.97	0.714	1.035	1:1.45	0.023	90
31	"	1.95	0.660	0.968	1:1.47	0.031	85
14	"	1.97	0.705	1.015	1:1.44	0.047	90
14	Xylose.	1.94	0.771	1.043	1:1.36	0.030	95
118-8*	"	1.41	0.545	0.732	1:1.34		91
52-7	"	1.94	0.780	1.082	1:1.39	0.021	97

\* From *J. Biol. Chem.*, 1919, xxxix, 368.

Aside from this somewhat lower ratio between acetic and lactic acids for the mannitol-forming group, no essential difference manifests itself in the splitting of the two pentoses by the different bacteria. The sugars are almost completely fermented in all cases; carbon dioxide is produced only in minute quantities, and acetic and lactic acids constitute almost the entire amount of measurable products. On the basis of these data the fermentation equation may be represented as:



A certain quantity of the sugar is incorporated in the cells of the bacteria and a small quantity is consumed for their development, but the foregoing equation is as nearly quantitative as can be expected of a biological process.

### *Identification of Products.*

*Volatile Acid.*—The volatile acid from the various fermentations was subjected to a Duclaux distillation and the distilling constant calculated from the titration data. The constants obtained are given in Table IX. For comparison the distilling constant obtained with our apparatus for acetic acid made from recrystallized barium acetate and Duclaux's original constant for acetic acid are also given in this table. The results indicate that the volatile acid is practically pure acetic acid in all cases, although the constants are slightly higher than for acetic acid. The difference is within the range of experimental error. Slight variation in the constants is to be expected with different pieces of apparatus, and different methods of heating. Additional evidence for the absence of a higher fatty acid such as propionic acid was obtained by fractionating a volatile acid distillate, Arabinose Culture 14, containing 200 cc. of 0.1 N acid. The barium salt was dissolved in 100 cc. of water, 50 cc. of 0.1 N sulfuric acid were added, and the partially freed volatile acid was distilled with steam. The higher fatty acids such as propionic and butyric would, if present, be concentrated in the distillate leaving the lower acids in the distilling flask. The fractional distillate was submitted to a Duclaux analysis, but proved to have the same distilling constant as the unfractionated distillate. Since there was no change in the distilling constant, it is evident that the first distillate contained a single volatile acid and not a mixture.

As a check on the Duclaux analysis the barium content of the volatile acid was determined. The barium salt was dried to constant weight in a platinum dish at 130°C. and then ignited with an excess of sulfuric acid. The weights of barium sulfate found and the calculated quantity that should have been present if the salt was barium acetate are given in Table X. The close agreement between the found and theoretical values proves that the volatile acid was acetic and corroborates the conclusions of the Duclaux analysis.

TABLE IX.  
*Distilling Constants of the Volatile Acids Obtained by Duclaux Method.*

Culture No.	Source of acid.	Fractions.									
		10 cc.	20 cc.	30 cc.	40 cc.	50 cc.	60 cc.	70 cc.	80 cc.	90 cc.	100 cc.
29	Arabinose.	7.5	15.4	23.7	32.4	41.4	51.0	61.2	72.1	84.9	100.0
29	Xylose.	7.5	15.5	23.8	32.5	41.6	51.1	61.4	72.6	85.1	100.0
124-2	Arabinose.	7.5	15.5	23.8	32.5	41.5	51.2	61.4	72.6	85.0	100.0
124-2	Xylose.	7.5	15.4	23.9	32.3	41.3	50.9	61.2	72.4	85.0	100.0
102	Arabinose.	7.4	15.5	23.8	32.4	41.4	50.9	61.3	72.4	85.1	100.0
31	"	7.6	15.5	24.0	32.7	41.8	51.4	61.7	72.8	85.3	100.0
14	"	7.6	15.6	24.0	32.8	41.9	51.5	61.7	72.9	85.3	100.0
14	" *	7.6	15.6	24.0	32.6	41.8	51.3	61.6	72.6	85.2	100.0
14	Xylose.	7.7	15.7	24.1	32.8	42.0	51.4	61.9	73.0	85.5	100.0
Author's constant for purified acetic acid.		7.4	15.2	23.6'	32.2	41.4	51.0	61.3	72.4	85.1	100.0
Duclaux constant for acetic acid.		7.4	15.2	23.4	32.0	40.9	50.5	60.6	71.9	84.4	100.0

\* First fraction of total acid used in preceding distillation—Arabinose Culture 14.



*Non-Volatile Acid.*—The barium salts were evaporated to dryness, taken up with 10 to 15 cc. of water, filtered, and absolute alcohol was added to the filtrate, as in the Möslinger method (25), until a concentration of 90 per cent alcohol was reached. In most cases a slight precipitate of some amorphous material was formed. A similar precipitate obtained by extracting the uninoculated control showed it to be an impurity from the yeast water and not the barium salt of some non-volatile acid produced by fermentation. A portion of the alcohol solution was evaporated to dryness and dried at 130°C., and the barium content of the residue determined as in the case of the volatile acid. The data are given in Table XI and agree satisfactorily

TABLE X.  
*Composition of Barium Salts of the Volatile Acids.*

Sugar fermented.	Culture No.	Barium salt of the volatile acid.	Barium sulfate equivalent.	
			Found.	Calculated for acetic acid.
		<i>gm.</i>	<i>gm.</i>	<i>gm.</i>
Arabinose.	29	0.5680	0.5132	0.5189
Xylose.	29	0.3128	0.2812	0.2858
Arabinose.	124-2	0.5983	0.5448	0.5466
Xylose.	124-2	0.7002	0.6370	0.6397
Arabinose.	102	0.3880	0.3530	0.3545
"	31	0.6910	0.6288	0.6314
"	14	0.7560	0.6890	0.6908
Xylose.	14	0.3354	0.3026	0.3065

with those required for lactic acid. The optical form of the lactic acid was ascertained by preparing the zinc lactate and determining its water of crystallization. Inactive zinc lactate crystallizes with 3 molecules of water, or 18.17 per cent, while the active form contains only 2 molecules, or 12.9 per cent. The percentages of water found are given in Table XII and prove that all of these different bacteria produce inactive lactic acid.

In previous publications (11, 12, 13) it has been shown that the mannitol-forming bacteria of Group II as far as has been determined always form a racemic mixture of lactic acid. This is true for the fermentation of both the pentoses and hexoses and also holds for mannitol. The non-mannitol-forming bacteria, Group

I, exhibit this same characteristic toward pentoses. The kind of lactic acid produced from hexoses and hexahydric alcohols remains to be determined.

TABLE XI.  
*Composition of the Barium Salts of the Non-Volatile Acids.*

Sugar fermented.	Culture No.	Barium salt of the non-volatile acid.	Barium sulfate equivalent.	
			Found.	Calculated for lactic acid.
		<i>gm.</i>	<i>gm.</i>	<i>gm.</i>
Arabinose.	29	0.3376	0.2456	0.2498
Xylose.	29	0.2756	0.2024	0.2039
Arabinose.	124-2	0.7104	0.5268	0.5257
Xylose.	124-2	0.5156	0.3750	0.3815
Arabinose.	102	0.8040	0.5852	0.5949
"	31	0.8996	0.6578	0.6657
"	14	0.3426	0.2464	0.2535
Xylose.	14	0.3232	0.2372	0.2392

TABLE XII.  
*Water of Crystallization Contained in the Zinc Lactates.*

Culture No.	Source of salt.	Weight of zinc lactate used.	Water lost.		Water in Zn (C <sub>3</sub> H <sub>5</sub> O <sub>3</sub> ) <sub>2</sub> + 3H <sub>2</sub> O.
			<i>gm.</i>	<i>per cent</i>	
29	Arabinose.	0.7466	0.1342	18.0	18.17
29	"	0.3854	0.0694	18.0	18.17
29	Xylose.	0.6024	0.1072	17.6	18.17
124-2	Arabinose.	1.2256	0.2220	18.1	18.17
124-2	Xylose.	1.1942	0.2162	18.1	18.17
102	Arabinose.	2.2206	0.4042	18.2	18.17
31	"	1.7082	0.3094	18.1	18.17
14	"	1.5862	0.2870	18.1	18.17
14	Xylose.	0.6864	0.1232	18.0	18.17

#### SUMMARY.

The pentose sugars, arabinose and xylose, are readily fermented by various strains of the lactic acid bacteria. These pentose-destroying bacteria are widely distributed in nature, occurring in large numbers in silage, sauerkraut, and related products. At different stages of the fermentation of corn silage and sauer-

kraut, pure cultures of these lactic acid bacteria were isolated and their general characteristics studied. It was found that these organisms are usually short, blunt ended rod forms occurring as single cells or long filaments. From a large number of cultures isolated 12 were selected for special study.

This choice of 12 cultures was based chiefly on the amount of acid formed from arabinose and xylose, on the change noted in litmus milk, and on the source of the culture. According to their behavior in milk the lactic acid bacteria which ferment pentoses may be arranged in two groups, the one group which slowly causes the milk to coagulate, the other which fails to bring about any noticeable change.

Because of the very slight variation in morphology, the separation of these organisms into groups depends upon characters other than cell structure. Measurements of the fermentative ability are undoubtedly the best means of separating the various groups and strains of the lactic acid bacteria. Among the compounds most valuable for differentiation are xylose, arabinose, fructose, lactose, melezitose, dulcitol, and  $\alpha$ -methyl glucoside. The organisms studied naturally fall into two great groups; those of Group I ferment fructose without forming mannitol, and those of Group II ferment fructose with the production of mannitol. Aside from the two main divisions these organisms may be arranged into several subdivisions or strains, depending upon differences in kind of sugars fermented and amount of acid formed.

The following group of reactions indicates the nature of these strains:

*Group I.*—Strain A ferments arabinose, xylose, and lactose, but does not ferment melezitose or dulcitol. Strain B ferments arabinose, xylose, lactose, and dulcitol, but does not ferment melezitose. Strain C ferments arabinose, lactose, and melezitose, but does not ferment xylose or dulcitol.

*Group II.*—All strains ferment arabinose and xylose, but do not ferment lactose, melezitose, or dulcitol.

No doubt some of these forms have been described previously; however, the characters reported are not in sufficient detail to insure identification.

The authors suggest the following names for these various types of lactic bacteria:

*Group I.*—Strain A—Cultures 29, 102-1, *Lactobacillus pentosus*, *n. sp.* Strain B—Cultures 124-1, 124-2, *Lactobacillus pentosus*, *n. sp.*

The authors feel that the difference between Strains A and B is not sufficient to warrant a separate name for Strain B.

Strain C—Cultures 102, 31, and 32. *Lactobacillus arabinosus*, *n. sp.*

*Group II.*—This group contains closely related organisms belonging to the *Lactobacillus pentoaceticus* type.

The fermentation of arabinose and xylose by certain of the lactic acid bacteria results in the production, mainly, of acetic acid and lactic acid. These two compounds are equivalent to about 90 per cent of the sugar destroyed and 98 per cent of the isolated products. The ratio of the two acids to one another is approximately 1 molecule of acetic to 1 molecule of lactic. The theoretical ratio of their molecular weights is 1:1.50, while the ratios found varied from 1:1.34 to 1:1.52. The mannitol-forming bacteria slowly ferment lactic acid to acetic acid and carbon dioxide. The secondary fermentation results in a deviation from the theoretical ratio in the direction of the lower values.

The only other product that could be identified was carbon dioxide. This is produced in minute quantities; from 10 to 20 mg. are formed per gm. of sugar fermented.

On the basis of the almost complete fermentation of the pentoses and the high percentage of sugar accounted for by the products, it appears that the main line of the fermentation is simple cleavage into acetic and lactic acids.

The pentose-fermenting organisms studied represent a closely related family which may be divided into groups and strains according to their fermentative ability. Although these organisms possess differential fermentation characters, the products in the breaking down of arabinose and xylose by them are identical, and in the same proportions.

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## EXPLANATION OF PLATES.

## PLATE 1.

FIG. 1. Culture 29. A 24 hour culture on arabinose yeast agar. Stained with fuchsin.  $\times$  1,000.

FIG. 2. Culture 124-2. A 24 hour culture on arabinose yeast agar. Stained with fuchsin.  $\times$  1,200.

## PLATE 2.

FIG. 3. Culture 102. A 24 hour culture on arabinose yeast agar. Stained with fuchsin.  $\times$  1,200.

FIG. 4. Culture 31. A 24 hour culture on arabinose yeast agar. Stained with fuchsin.  $\times$  1,000.

FIG. 5. Culture 14. A 24 hour culture on arabinose yeast agar. Stained with fuchsin.  $\times$  1,000.



FIG. 1.



FIG. 2.

(Fred, Peterson, and Anderson: Pentose-destroying bacteria.)







FIG. 3.



FIG. 4.



FIG. 5.

(Fred, Peterson, and Anderson: Pentose-destroying bacteria.)



## THE EXCRETION OF ACETONE FROM THE LUNGS.

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(Received for publication, July 18, 1921.)

It is well known that under conditions which lead to the formation and accumulation of the "acetone bodies," acetone is present in the breath. It occurred to one of us that this excretion of acetone from the lungs might be the result merely of evaporation from the blood plasma into the alveolar air, conditioned by the distribution coefficient of acetone between plasma and air at the body temperature. To learn whether this surmise was correct we have determined the distribution coefficients of acetone between water and air, and blood and air outside the body at different temperatures, and with these data have compared results, and coefficients obtained therefrom, from human subjects of natural ketonemia and from dogs after the injection of acetone solutions intravenously.

Our experiments lead to the conclusion that acetone is excreted from the lungs by the simple process of diffusion and volatilization, since the ratio between the concentrations in blood and alveolar air is the same as the distribution coefficient determined *in vitro* at body temperature. A smaller number of experiments indicate that the concentration of acetone in urine is equal to and parallels its concentration in blood, and the excretion of acetone by the kidneys also thus appears to be the result of simple diffusion.

At the time of beginning our experiments about 2 years ago, we were under the impression that the question had not been taken up from the point of view mentioned, but this impression proved to be incorrect when the literature was more carefully searched.

The characteristic odor of the breath of patients showing symptoms of diabetic coma, and the fact that this odor is due at

least in part to acetone, noted by Petters (1) in 1857, is the beginning of practically all of our knowledge concerning the acetone bodies. Although most of the host of workers who have contributed to the subject have devoted their attention to the excretion of these substances by the kidneys there have been many observations on the excretion of acetone by the lungs. The first quantitative determinations of the amount thus excreted seem to have been made in 1897. In that year Nebelthau (2) recorded the excretion of 150 mg. per hour by the lungs by a subject of chronic inanition who excreted about one-tenth that amount in the urine. The same year Geelmuyden (3) found that on giving acetone by mouth or subcutaneously to rabbits and dogs it was in great part excreted by the lungs; and Schwarz (4) showed that dogs and rabbits excrete only a few per cent of injected or fed acetone in the urine while from 50 to 76 per cent appears in the breath. In the same volume in which Schwarz' paper was published, Müller (5) describes and pictures a simple apparatus with which he determined the amount of acetone excreted in the breath. The exhaled air was bubbled through ice water and the absorbed acetone titrated by the Messinger method. With normal well fed subjects he found 1.3 to 3.3 mg. of acetone per hour, with diabetics up to 20 mg. per hour, and after giving by mouth 3.8 gm. of acetone to a normal subject, 130 mg. per hour in the exhaled air. Although he records no blood analyses and no individual determinations, he concluded that the amount of acetone in the breath depends upon (1) the content of acetone (or acetoacetic acid) in the blood or tissues, and (2) the volume of respired air, the evaporation in the lung capillaries being faster the lower the acetone tension in the alveolar air and *vice versa*. It is evident that Müller had in mind that the pulmonary excretion of acetone is determined by its partial pressure in blood and alveolar air, though he offered no data to prove that such is the case. In 1910 Cushny (6), without it seems being aware of Müller's work, reported data from three experiments on cats which had received acetone intravenously, showing that the excretion in the breath was fairly constant over a 3 hour period, from 8.5 to 14 per cent being excreted within that time, depending upon the amount injected. He states that the "percentage of that injected which is excreted in a given time

thus rises with the dose." He also performed one experiment in which measured volumes of air were drawn through an acetone solution (containing chloroform as well) of known concentration and found that the rate of evaporation of acetone was similar to the rate of its excretion in exhaled air by a cat which had been injected with acetone solution. Although he had from these observations data from which the distribution coefficient of acetone might have been calculated approximately, the results were not considered from this point of view. Indeed near the close of his paper, in discussing the reason for the different rates of exhalation of chloroform, methyl and ethyl alcohol, and acetone, he suggests that the different behavior is determined by their *solubility* in and *miscibility* with plasma, and states that "acetone while completely miscible with water is not so nearly related in constitution and its evaporation and exhalation are thus *less dependent on its concentration in the blood.*" We find, on the contrary, that the rate of evaporation of acetone, at any rate, and its exhalation from the lungs is directly dependent upon its concentration in the blood, which in turn determines the partial pressure in the (alveolar) air with which the solution (blood) is in equilibrium. Acetone in dilute aqueous solutions, in the body as well as *in vitro*, appears to behave in accord with Henry's law and it is perhaps not unlikely that such other substances as chloroform and alcohol would be found to behave in the same way. Cushny, nevertheless, concluded that the "exhalation of volatile substances from the lungs is exactly analogous to their evaporation from solutions in water," which as regards acetone is confirmed by our results.

Finally, in a recent paper Widmark (7) has reported experiments covering almost the identical ground as our own and leading to the same conclusions. He determined the distribution coefficient of acetone between water and air at 38°C. and found the ratio,  $\frac{\text{concentration in solution}^1}{\text{concentration in air}}$  to be 406 and 389 by two different methods. In one case only the concentration in air,

<sup>1</sup> Widmark gives the ratio  $\frac{\text{air}}{\text{solution}}$ , the reciprocal of the relation, which we have preferred to use. We have recalculated his data for comparison with our own.

and in the other only the concentration in solution was determined, the other concentration being calculated or assumed in both cases. For blood serum he gives 392 and for whole beef blood 323 and 304 as values of the same ratio. On making simultaneous analyses of blood and alveolar air of a normal subject after taking various amounts of acetone by mouth, he found as values of  $\frac{\text{concentration in blood}}{\text{concentration in alveolar air}}$  from 394 to 334, thus demonstrating approximately the same relationship as found *in vitro* with blood. In another paper Widmark (8) showed that the concentration of acetone in urine very closely parallels its concentration in blood and that the amount excreted by the kidneys in a given time thus depends only upon the blood content and the urine volume.

*Distribution of Acetone between Water and Air, in Vitro.*

For the determination of the distribution coefficient of acetone we have used several different procedures of which only one need be described in detail. In all cases we have directly determined by the iodoform titration the amount of acetone both in the air and in the solution with which the air was in equilibrium. In one method about 2 liters of air were shaken with about 500 cc. of acetone solution in bottles at room temperature (laboratory, warm room, and cold room), and in another a measured volume of air was bubbled through successive tubes of acetone solution placed in a water thermostat. Observations were made over a considerable range of temperature and with solutions of different concentration.

The arrangement of apparatus usually used is shown in Fig. 1. Tubes, *A*, *B*, and *C* and the bottle, *D*, were nearly filled with the same acetone solution and were covered by the water of the thermostat which was stirred and maintained within  $0.1^{\circ}$  of the desired temperature. A thermometer in the bottle indicated the temperature at which the air and solution were in final equilibrium, and an additional exit tube was connected with a water manometer (*E*), to insure atmospheric pressure. Air was bubbled through, using just enough pressure at the inlet and suction at the outlet to maintain a steady flow at atmospheric pressure

in the bottle, *D*. Two tubes each containing about 60 cc. of cold water, and surrounded by water and ice, outside the thermostat, absorbed the acetone from the air,<sup>2</sup> the volume of which was measured at room temperature by the water displaced from the siphon bottle, *F*. The volume was calculated to the temperature of the bottle, *D*, at which equilibrium was established, making correction for temperature and water vapor. Usually about 2 liters of air were taken for a determination. Samples of the solution were withdrawn from the bottle, *D*, from time to time and analyzed. The concentration in *D* changes very

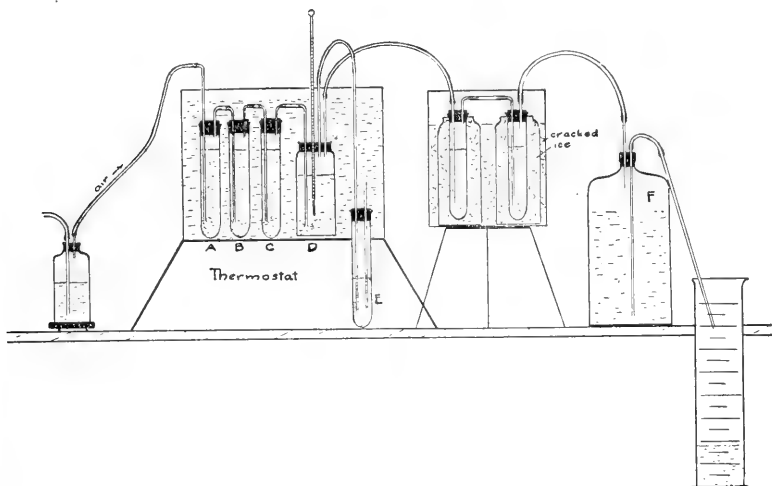


FIG. 1. Apparatus used for determining distribution coefficient of acetone between solution and air.

slowly since the air receives its acetone from the earlier tubes. The following example illustrates the data determined and the method of calculation.

<sup>2</sup> That the acetone was for practical purposes completely absorbed in the two tubes of ice water was proved by the following experiment. 4 liters of air passed through the tubes of the thermostat which contained an acetone solution of approximately 0.1 per cent. The acetone was collected in a series of three tubes of ice water (4.0°C.). The acetone in the first two tubes was determined by titration to be 42.7 mg. The amount of acetone in the third tube was determined nephelometrically to be 0.0118 mg. or about 0.003 mg. per liter of air. The ratio  $\frac{\text{solution}}{\text{air}}$  at 5° is about 1,900.

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Temperature (of solution during equilibration) = 37.8°C. Barometric pressure = 750. 2,000 cc. of air measured at 27.4°C. contained 6.85 mg. of acetone. 10 cc. of acetone solution from the last tube in the thermostat contained 10.64 mg. of acetone, or 1,064 mg. per liter.

(Vapor tension, H<sub>2</sub>O at 27.4° C. = 27 mm. at 37.8° = 49 mm. of Hg)

$$2,000 \times \frac{273 + 37.8}{273 + 27.4} \times \frac{750 - 27}{750 - 49} = 2,134 \text{ cc. at } 37.8^\circ$$

$$\frac{1,000}{2,134} \times 6.85 = 3.21 \text{ mg. of acetone per liter of air.}$$

$$\text{Ratio of concentrations, } \frac{\text{solution}}{\text{air}} = \frac{1,064}{3.21} = 332.$$

The interest being in the values of the distribution coefficient at body temperature, only the results between 37° and 38°C. are given (Table I). These results and many others at higher and lower temperatures show that the ratio between solution and air is quite independent of concentration over a wide range.

The distribution coefficient was determined also for blood serum (beef) with added acetone using an additional tube with glass-wool as a trap to catch foam. It will be seen that the values are practically the same as with pure solutions of acetone.

The average of results give the following values:

$$37\text{--}38^\circ \frac{\text{solution}}{\text{air}} = \text{for pure solutions, } 334; \text{ for serum, } 337.$$

A few determinations were made also with defibrinated blood (beef), but more difficulty was experienced from foaming, and the results were not quite reliable. They indicated roughly the same values as obtained with serum.

*The Ratio of Acetone in Blood and Alveolar Air.*—In order to determine acetone in alveolar air it was desired to have the animals or subjects rebreathe into a bag as in the Plesch method for CO<sub>2</sub>. A rubber bag was found unsuitable due to a rapid loss of acetone vapor, probably by solution in the rubber. The concentration of acetone in the air decreased about 30 per cent in a half hour.

A satisfactory bag was made from sheets of adhesive plaster, to the gummed side of which glazed tracing paper was attached. Two sheets of this fabric 8 by 10 inches were glued and clamped together along three sides; into the open end of the bag thus formed, a covered rubber stopper carrying a glass outlet tube was fitted and glued in place. In order to absorb moisture from expired



air, and prevent its condensation inside the bag, a short calcium chloride tube was attached to the outlet tube of the bag, fresh lumps of  $\text{CaCl}_2$  being used for each experiment. When air was bubbled through an acetone solution of known concentration in the thermostat apparatus into this bag and subsequently drawn from the bag through ice water, the amount of acetone

TABLE I.  
*Distribution of Acetone between Solutions and Air.*

Temperature.	Acetone per liter.		Ratio.
	Solution.	Air.	$\frac{\text{Solution}}{\text{Air}}$
Acetone in distilled water.			
	mg.	mg.	
37.0	2.143	6.65	323
37.1	2.143	6.74	318
37.7	1.220	3.67	332
37.8	1.120	3.43	328
37.2	1.115	3.29	339
37.3	255	0.748	341
37.3	1.064	3.07	347
37.5	1.064	3.08	346
37.9	1.064	3.20	333
37.8	1.064	3.23	329
37.8	5.326	16.06	332
Average.....37.50			334
Acetone in blood serum.			
37.6	3.020	8.69	347
37.0	3.020	9.22	328
37.6	3.020	9.12	332
37.4	3.020	8.99	336
Average.....37.4			337

obtained from a measured volume was in agreement with that computed from the concentration of the solution and the distribution coefficient at the observed temperature. With this bag, thus shown to be relatively impermeable to acetone vapor, we determined the concentration of acetone in the alveolar air of a number of human subjects and of experimental animals.

After rebreathing from the bag for a period of 30 to 60 seconds, the air was passed through two tubes of ice water, surrounded by water and ice, the volume of air being measured as in the thermostat experiments at room temperature and calculated to its volume, moist, at body temperature. The acetone absorbed by the tubes of ice water was determined by titration with 0.01 or 0.002  $\times$  iodine and thiosulfate.

The determination of acetone in blood was accomplished by aerating the Folin-Wu blood filtrates. Oxalated blood was precipitated by tungstic acid according to the Folin-Wu directions (9), using small flasks to minimize the volume of air in contact with the solution. The mixtures were filtered on funnels with long stems, and covered by watch-glasses to minimize loss during filtration. 10 cc. of the filtrate, equivalent to 1 cc. of blood, were measured into large test-tubes containing about 5.0 gm. of NaCl, and the solution was aerated into another large test-tube containing 50 cc. of water, 2 cc. of 5  $\times$  NaOH, and 10 cc. of 0.002  $\times$  iodine. After 10 minutes of slow aeration, an additional 10 cc. of iodine were added, and the rate of aeration increased and continued 15 minutes. Normal blood to which were added known amounts of acetone, when analyzed under these conditions, gave results within 2 or 3 per cent of the calculated value.<sup>3</sup>

#### *Experiments on Dogs after Injection of Acetone Solutions.*

*Experiment 1.*<sup>4</sup>—A female dog of 7.5 kilos was given 4 gm. of urethane in 200 cc. of water by mouth. A tracheal cannula was inserted under local anesthesia (benzyl alcohol). The bladder was emptied by catheter and control samples of blood and alveolar air were taken, after which 75 cc. of 14.5 per cent acetone solution (10.87 gm. or 1.45 gm. per kilo of body weight) were injected intravenously. Samples of blood, alveolar air, and urine were collected as stated in the protocol.

10.21 a.m. Control air, 760 cc. at 28° = 805 cc. at 37.6° = 0.046 mg. of acetone. Control urine, 13 cc. 10.24 a.m. Bladder emptied. 10.27 a.m. Blood control. 10.50 a.m. 75 cc. of 15 per cent acetone solution intravenously. 10.52 a.m. Urine 1 = 3.3 cc. 10.59 a.m. Air 1 = 680 cc. at

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<sup>3</sup> Air under pressure was used in aeration. After 30 minutes aeration, 20 cc. of 0.01  $\times$  iodine showed a loss of about 0.15 cc. and 20 cc. of 0.002  $\times$  iodine a loss of about 0.25 cc.

<sup>4</sup> The thanks of the authors are due Dr. E. K. Marshall, Jr., and Dr. W. H. Olmsted for assistance in conducting several of the experiments.

28° = 720 cc. at 37.6° = 5.02 mg. of acetone. 11.00 a.m. Blood 1 = 15 cc. 11.06 a.m. Urine 2 = 5.2 cc. + 20 cc. of wash water. 11.11 a.m. Rectal temperature = 37.6°. 11.15 a.m. Air 2 = 650 cc. at 28° = 689 cc. at 37.6° = 4.41 mg. of acetone. 11.24 a.m. Blood 2. 11.28 a.m. Air 3 = 660 cc. at 28° = 700 cc. at 37.6° = 4.07 mg. of acetone. 11.33 a.m. Blood 3. 11.35 a.m. Urine 3 = 21 cc. + 20 cc. of wash water. 11.45 a.m. Air 4 = 730 cc. at 28° = 774 cc. at 37.6° = 4.38 mg. of acetone. 11.48 a.m. Blood 4. 11.58 a.m. Urine 4 = 10 cc. + 20 cc. of wash water. 12.02 p.m. Air 5 = 650 cc. at 28° = 689 cc. at 37.6° = 3.63 mg. of acetone. 12.06 p.m. Blood 5. 12.14 p.m. Urine 5 = 6.5 cc. + 20 cc. of wash water. 12.28 p.m. Air 6 = 750 cc. at 28° = 795 cc. at 37.6° = 3.84 mg. of acetone. 12.30 p.m. Blood 6. 12.37 p.m. Urine 6 = 3.5 cc. + 20 cc. of wash water. 12.56 p.m. Blood 7. 1.00 p.m. Air 7 = 695 cc. at 28° = 737 cc. at 37.6° = 3.62 mg. of acetone. 1.06 p.m. Urine 7 = 6 cc. 2.01 p.m. Blood 8. Air 8 = 688 cc. at 28° = 729 cc. at 37.6° = 3.39 mg. of acetone. 2.02 p.m. Urine 8 = 5.8 cc.

The analytical results of this experiment are given in Table II and have been plotted in time curves shown in Fig. 2. For the calculation of the ratios between concentration of acetone in blood and in alveolar air, given in the last column of the table, a curve of the concentration in blood plasma was plotted and values (in parentheses in table) read off corresponding to the time of taking the air samples. The ratio of concentrations  $\frac{\text{plasma}}{\text{air}}$  varies from 320 to 350, the average being 336, or nearly

the same as observed in the *in vitro* experiments (337). The variation is irregular and is doubtless due to accumulated errors in the procedure. The curves show that the concentration of acetone in both alveolar air and urine follows very closely that in blood, the amount in urine and blood being almost identical. The initial rise in the urine is probably in response to a much higher concentration in the blood immediately after the injection and before the first blood sample was drawn. With the fall of the amount in blood due in large part to its passage into the tissues, the concentration in urine fell to or slightly below that in blood.

It is of interest to learn the relative amounts of acetone excreted by this animal from the lungs and kidneys. The total urine secreted during 3 hours and 10 minutes was 61 cc., containing 114 mg. of acetone. The total excretion by the lungs was

not determined but may be very roughly calculated as follows. The respirations varied from 13 to 25 per minute of from 100 to 130 cc., giving a total volume per minute of  $18 \times 115 = 2,070$  cc. The alveolar air may be taken as containing about 5 mg. per liter and the tidal air perhaps 4.0 mg. per liter. This gives a total excretion by the lungs during the experiment of 1.56

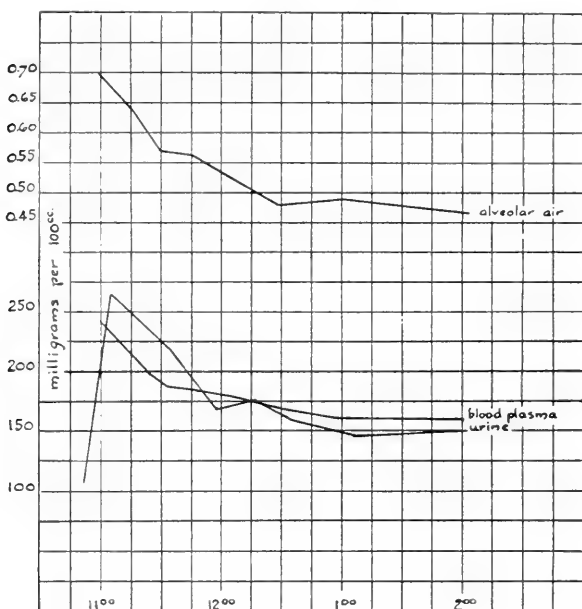


FIG. 2. Curves showing parallel changes in concentration of acetone in blood, alveolar air, and urine of dog after intravenous injection (Experiment 1).

gm. or roughly fifteen times the excretion by the kidneys during the same time. Since the concentration in urine is independent of the urine volume, the amount thus excreted will, of course, depend upon the amount of urine secreted as well as upon the blood concentration. And similarly the amount excreted in a given time by the lungs is determined by the volume of respired air which aerates the acetone from the blood.

TABLE II.  
*Experiment 1.*

Time.	Acetone per 100 cc.				Ratio.
	Whole blood aerated	Blood plasma aerated.	Urine.	Alveolar air.	Blood plasma Alveolar air
<i>a. m.</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>	
10.21	1.8			0.0057	
10.27					
10.50	Acetone injected.				
10.52			106		
10.59		(242)		0.698	347
11.00	236	242			
11.06			264		
11.15		(205)		0.640	320
11.24	198	196			
11.28		(194)		0.571	340
11.33	188	191			
11.35			217		
11.45		(185)		0.566	327
11.48	186	184			
11.58			168		
<i>p. m.</i>					
12.02		(179)		0.527	340
12.06	177	176			
12.14			175		
12.28		(170)		0.483	350
12.30	163	169			
12.37			159		
12.56	165	161			
1.00		(160)		0.491	326
1.06			* 147		
2.01	163	159		0.465	342
2.02			151		
Average .....					336

*Experiment 2.*—Under ether anesthesia a tracheal cannula was inserted in a dog weighing 10 kilos. Urethane was injected intraperitoneally and the ether discontinued. Blood and air samples were taken before and after the injection of acetone solution as stated in the protocol. The analytical results are given in Table III.

2.45 p.m. 10 gm. of urethane intraperitoneally. 3.00 p.m. Control samples of blood and alveolar air. 3.05 p.m. 25 cc. of acetone solution

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(50 per cent) injected into the peritoneal cavity. 3.23 p.m. Blood sample 1. 3.27 p.m. Air sample 1 = 395 cc. at 27°. 3.55 p.m. Blood sample 2. 4.00 p.m. Air sample 2 = 410 cc. at 27°. 4.10 p.m. Blood sample 3. 4.14 p.m. Air sample 3 = 330 cc. Rectal Temperature 97°. 4.25 p.m. Blood sample 4. 4.30 p.m. Air sample 4 = 400 cc. at 27°. 4.40 p.m. Blood sample 5. 4.43 p.m. Air sample 5 = 295 cc. at 27°. 5.00 p.m. Blood sample 6. 5.05 p.m. Air sample 6 = 380 cc. at 27°. 5.15 p.m. Blood sample 7. 5.25 p.m. Air sample 7 = 520 cc. at 27°. 5.35 p.m. Blood sample 8. 5.40 p.m. Air sample 8 = 250 cc. Rectal Temperature = 97°. Dog killed with chloroform.

TABLE III.  
*Experiment 2.*

Time.	Acetone per 100 cc.		Ratio.
	Blood serum.	Alveolar air.	
<i>p. m.</i>	Acetone solution injected (25 cc. of 50 per cent).		$\frac{\text{Concentration of serum}}{\text{Concentration of alveolar air}}$
3.05	157		
3.23	(156)		
3.27	(156)	0.453	344
3.55	149		
4.00	(145)	0.471	307
4.10	132		
4.14	(131)	0.390	336
4.25	128		
4.30	(127)	0.392	324
4.40	125		
4.43	(125)	0.377	329
5.00	123		
5.05			
5.15	114	0.328	369
5.25			
5.35	113	0.368	310
5.40			
		0.357	317
Average.....			330

The ratios  $\frac{\text{serum}}{\text{alveolar air}}$  vary considerably, from 307 to 369, the average being 330. It will be noted that the acetone (12.5 or 0.8 gm. per kilo) was injected into the peritoneal cavity from which it rapidly passed into the blood and from the blood was more slowly distributed and excreted.

Four other experiments of similar character yielded results which gave ratios,  $\frac{\text{blood}}{\text{alveolar air}}$ , from about 280 to 380, most of which were of doubtful accuracy and for that reason will not be recorded. The acceptable results leave no doubt that distribution of acetone between blood and air in the lungs of dogs is substantially the same as the distribution found *in vitro*.

*Distribution of Acetone between Blood and Alveolar Air in Diabetic Acidosis.*

The determinations in blood and alveolar air were carried out as above described, at once after collecting samples. The results obtained are given in Table IV in which is also included for comparison a summary of the results on animals and a few of the results of *in vitro* experiments. Results are recorded also of two determinations on normal subjects on the morning of the third day of fast, when acetone bodies were being formed in small amounts. For the determinations of acetone in alveolar air of these normal subjects 5 liters of air, separately equilibrated by rebreathing 1 liter portions, were taken for analysis. The ratio between blood and air was evidently the same as in the diabetic subjects with marked acidosis.

These results from diabetic and normal subjects show conclusively that the concentration of acetone in alveolar air bears a constant relationship to the concentration of free, preformed acetone in the blood, and that this relationship is expressed by the distribution coefficient of acetone between the air and its solution in blood plasma. From this fact it follows that one may learn the amount of acetone in blood by determining the amount in alveolar air and multiplying the result by a factor, the value of which according to our data is about 340. And if there were also a constant relation between the amount of acetone, and of the related acetoacetic and hydroxybutyric acids it would be possible to calculate also the latter values. In Table V are given the results on a few blood analyses showing separately the amounts of acetone, and acetoacetic and hydroxybutyric acids.

Although these results are too few to justify generalization, they indicate that the relative amounts of free acetone in blood

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vary at least from 13 to 26 per cent of the sum of acetone, and acetoacetic and hydroxybutyric acids. One may, therefore, perhaps get a rough approximation of the amount of total acetone bodies (expressed as acetone) present in blood by multiplying the concentration in alveolar air by, say 1,700, ( $340 \times \frac{1.60}{2.0}$ ). Such a calculation is not to be recommended as a substitute for

TABLE IV.

*Summary of Results, Ratio of Acetone in Blood and Alveolar Air, and Comparison with Results in Vitro.*

Subject.	Condition.	Acetone per liter.		Ratio
		Blood.	Alveolar air.	$\frac{\text{Blood}}{\text{Air}}$
		Whole blood.		
		mg.		
P. R.	Diabetic acidosis.	112	0.324	345
O. L.	" "	96	0.278	345
F. M.	" "	148	0.436	340
Peters.	" "	199	0.560	355
K.	Normal, 3 days of fast.	64	0.163	392
C.	" 3 " " "	23	0.066	350
	Acetone injection.	Blood plasma.		
Dog.	Experiment 1.	2,420 to 1,590	6.98 to 4.65	336
"	" 2.	1,570 to 1,130	4.53 to 3.57	330

### *In vitro* experiments.

Water solutions.	Solution.	Air.	$\frac{\text{Solution}}{\text{Air}}$
Maximum concentration. ....	5,326	16.06	332
Minimum " .....	255	0.748	341
Average.....			334
Blood serum.....	3,020	8.96	337

the direct determination in blood. The excretion of acetone by the lungs is nevertheless of real value as affording a very simple means of detecting and roughly determining the extent of ketosis. The subject is asked merely to exhale for 1 or 2 minutes through a glass tube into a large test-tube of ice cold distilled water. At the end of the period of exhalation 10 or 20 cc. of Scott Wilson reagent are added and if ketosis exists a faint to deep opalescence



or precipitate appears after a few moments, the amount being roughly proportional to the amount of acetone exhaled. This test is quite sensitive and appears even before the urine shows a positive reaction with ferric chloride for acetoacetic acid.

TABLE V.  
*Acetone Bodies in Blood.*

No.	Subject.	As acetone in 100 cc. of blood.				Per cent of total.			Total acetone bodies in 24 hrs. as acetone in urine.	Remarks.
		Acetone.	Acetoacetic acid.	Hydroxybutyric acid.	Total.	Acetone.	Acetoacetic acid.	Hydroxy butyric acid.		
gm										
1*	"K," Severe diabetic.	17.6	10.4	45.2	73.2	24	14	62	56.7	Not in coma.
2*	" " "	3.3	7.7	13.5	24.5	14	31	55	15.9	" " "
3*	" " "	11.0	7.0	49.5	67.4	16	10	74	22.1	" " "
4*	Infant, 2 yrs.	3.8	9.5	15.8	29.1	13	33	54		" " " pyelitis.
5	"P," Severe diabetic.	19.9	15.6	42.5	78.0	25	20	55	27.1	Not in coma.
6	" " "				100.0			37		Death, not in coma, few hours later.
7	"W" Severe diabetic.	55.6	39.0	184.0	278.0	20	14	66	41.1	Death in coma, same day.
8	"S" Severe diabetic.				77.0			46	6.7	In light coma.
9	" " "				136.0			40	11.2	Death next day.

\* These results were previously reported from this laboratory by Marriott (Marriott, W. McK., *J. Biol. Chem.*, 1914, xviii, 515).

#### SUMMARY AND CONCLUSIONS.

1. The distribution coefficient of acetone for water and air in the vicinity of 37° and 750 mm. has been determined by two methods and found to be about 334 for  $\frac{\text{water}}{\text{air}}$ .

By the same technique the distribution coefficient of acetone for blood serum and air was found to be about 337.

2. The distribution of acetone in whole blood, blood plasma, blood serum, urine, and alveolar air of dogs which had been injected with large doses has been determined. The results show that the concentration of acetone in urine is about the same as that of whole blood and blood plasma and that the ratio of acetone in blood to that in alveolar air is about 333.

The distribution of acetone between alveolar air and blood of human diabetics and of normal fasting subjects was also determined and found to average 355.

From the above data it is concluded that acetone is excreted from the lungs and kidneys by the physical process of diffusion, thus confirming the recent observations of Widmark.

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## CARBOHYDRATE CONTENT OF THE KING SALMON TISSUES DURING THE SPAWNING MIGRATION.\*

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(Received for publication, August 10, 1921.)

The facts reported in this paper were developed as a part of the series of physiological studies on the king salmon, *Oncorhynchus tshawytscha*, during the summer of 1906. This salmon feeds in the California coastal waters to maturity and then migrates through San Francisco Bay and up the Sacramento River to spawning beds in the cold snow-fed waters of the McCloud River on the slopes of Mt. Shasta. The journey requires from 60 to over 100 days and is made without food.

The source of the energy used in salmon migration has long been a physiological problem of peculiar interest. Miescher studied the migrating Rhine salmon. Paton and others have published extensive papers on the chemical changes in the Scottish salmon, but carbohydrate metabolism has received little or no consideration.

It is not known to what extent the carbohydrates enter in the processes which liberate the dynamic energy expended in migration. The king salmon stores enormous percentages of fat, as in fact do all the salmon species thus far examined. This fat disappears during the migration and is probably the chief source of energy.<sup>1</sup> Kilborn and Macleod<sup>2</sup> have recently given analytical data to show that the tissues of certain marine invertebrates and fishes are low in glycogen, or its equivalent carbo-

\* Published by permission of the United States Commissioner of Fish and Fisheries.

<sup>1</sup> Greene, C. W., *Bull. U. S. Bureau Fisheries*, 1913, xxxiii, 69.

<sup>2</sup> Kilborn, L. G., and Macleod, J. J. R., *Quart. J. Exp. Physiol.*, 1918-20, xii, 317.

hydrate. In the single salmonoid reported, the lake trout, he found only a trace of carbohydrate in the muscle tissue.<sup>3</sup>

Our studies were made to supplement the general chemical studies by measurement of the glucose content of the tissues of the king salmon at different stages of the migration. Granting that the kinetic energy expended during the migration is derived primarily from the oxidations of the fats, still carbohydrates might play a significant part. Also, the developing ovaries store large amounts of food material in the yolk (the mature salmon egg is 6 to 7 mm. in diameter, hence contains 113 or more c.mm. of yolk). Carbohydrate is often present in the yolk of other eggs and should be found in the salmon eggs, at least when they are mature at the spawning.

Analyses were made of the following tissues: muscle, liver, ovary and ripe eggs, testes, skin, and stomach-intestine. Fishes of the series were chosen from the feeding grounds at Monterey Bay and Bolinas Bay,<sup>4</sup> Black Diamond, at the head of tide-water at the entrance of the Sacramento River into San Francisco Bay,<sup>5</sup> and at Baird on the spawning grounds on the McCloud River in the mountains of northern California.

#### *Method.*

The tissues or organs were ground fine in a meat chopper, the samples were weighed and preserved in 95 per cent alcohol in wide mouthed 240 cc. glass-stoppered bottles. The samples not immediately analyzed were sealed and shipped to the chemical laboratories at Stanford University.<sup>6</sup> The concentration of alcohol was that used in preserving other analytical samples which have been demonstrated to undergo no autolytic change. The samples

<sup>3</sup> The first announcement of the present data was made by the author during the discussion of the preliminary paper of Dr. Macleod presented at the Cincinnati meeting of the American Physiological Society, December 29, 1919.

<sup>4</sup> These fish are considered as the nearest representatives of the fully mature salmon entering the Golden Gate.

<sup>5</sup> No glucose analyses were obtained of samples from this station.

<sup>6</sup> The writer is under deepest obligation to Professor Lyonel R. Lenox of Stanford University for his cooperation and assistance in securing the chemical determinations.

Percentage of Glucose in Salmon Tissues of Fish Taken at Different Stages of Migration.

TABLE I.

Station.	Date.	Fish No. and sex.	Total length. mm.	Weight of salmon. gm.	Muscle. per cent	Liver. per cent	Ovary. per cent	Testes. per cent	Skin. per cent	Stomach. per cent
Monterey Bay.	1906									
	June 15	623 ♀	876	9,700		0.39				
	" 23	690 ♂	820	7,200		0.61				
	" 26	691 ♂	898	10,650		0.32				
	" 29	694 ♂	1,000	14,334		0.66				00
Bolinas Bay.	July 18	725 ♀	860	10,000		0.19				
	" 20	732 ♀	867	9,050	0.015 0.014	0.19 0.26	0.18			Trace.
	July 26	754 ♀	906	10,700	Trace.	0.70	0.09			
	" 26	765 ♂	890	11,350	"	0.21			0.038	0.041
	" 26				"	0.18				
McCloud River.	Aug. 15	810 ♀	745	5,200	00	0.08	0.072 0.069			
	" 15	821 ♀	814	6,650	00		0.08 0.09		00	
	" 16	850 ♂	1,046	9,350	00	0.123		00	00	
	" 28	938 ♀	915	8,450	00	0.026 0.021	0.09 0.07			
	" 28	939 ♂	875	7,300		0.110		00	00	
	" 28	940 ♂	890	6,300	00	0.017				
	" 30	946 ♀	878	7,650	00	0.020				
	" 4	975* ♂	948	9,800	00	0.019	0.09, eggs.	00		

\* Fine, dark-colored, immature, and pink meat fish.

were analyzed by the method of Pflüger and the glycogen was hydrolyzed and determined as glucose by the copper sulfate colorimetric method. The hydrolysis was used to guard against any loss by possible autolysis occurring during the preservation. The determinations are comparatively few but we have had no opportunity to add to the series. There are data enough in Table I, which gives the entire set of determinations for the series of samples, to indicate the average glycogen content of salmon tissues and the contrasts at the extremes of the fast.

#### *Muscle.*

Samples were taken of the great lateral muscle in a vertical band in the region of the anterior dorsal fin. In Fish 732, Monterey Bay, July 18, the duplicate samples show 0.015 and 0.016 per cent of glucose. These are voraciously feeding fish and are to be contrasted with Fishes 754 and 765 from Bolinas Bay near the mouth of the Golden Gate. The Bolinas fish are in the very prime of condition with the high store of 18 per cent of muscle fats.<sup>7</sup> They represent the highest nutritive value of any fish in this series, greater than the average for feeding fish at Monterey and of higher value than the migrating fish at tide-water on the Sacramento River. Fish at Bolinas Bay at this time had practically ceased feeding (judged by the absence of food content in the stomach).

Muscle samples were not obtained for glucose analysis from the Black Diamond fish.

The muscles of spawning fish are without glucose as shown by the analyses from four females and three males. Not a single sample yielded glucose. The tissues were perfectly fresh, often alive when minced.

#### *Liver.*

The salmon livers in fish from the Monterey feeding grounds show a variable glucose content, from 0.19 to 0.66 per cent. Livers from these fish examined histologically were crowded with fat droplets. Chemical analyses showed fat as high as 16.6 per cent. The glucose content is from ten to forty times greater than that

<sup>7</sup> Greene, C. W., *Tr. Am. Fisheries Soc.*, December, 1915.

of the muscle in the Monterey fish. The two Bolinus Bay fish vary as much as the extremes of the six examined at Monterey; *i.e.*, 0.18 and 0.70 per cent of glucose.

Samples of livers from six fish were analyzed from Baird. These livers from spawning fish contain with two exceptions less than 0.1 per cent of glycogen. The average is 0.057 per cent. This average is strikingly lower than the glycogen content of the Monterey or Bolinus Bay fishes. It indicates a depletion of the glycogen and a decrease of the part played by carbohydrate in liver metabolism during the fast.

#### *Ovaries and Eggs.*

The ovaries of the series of six fish are remarkably uniform in glucose content, whatever the stages of the journey. The percentages are from 0.08 to 0.18. This is a glucose content not above the average found in vertebrate blood.<sup>8</sup> One fish, No. 946, was a mature female from which spawning eggs were obtained. The analysis of these eggs, free from ovarian tissue and fluids, gave 0.09 per cent of glucose. This is near the average for the ovary of the series of immature fish. It demonstrates that the carbohydrate content in this growing tissue is independent of the stage of development and of the duration of the fast.

Tichomiroff<sup>9</sup> has given analyses of invertebrate eggs showing that in the eggs of *Bombyx* the glycogen amounts to 1.98 per cent. The total dry substance in these eggs is 35.51 per cent, somewhat less than salmon eggs, which average 45 per cent or more in total solids. Octopus eggs have been examined by Henze,<sup>10</sup> who found as high as 1 per cent of glucose in the fresh eggs (5.4 per cent in the dry residue). Kojo<sup>11</sup> found 0.272 per cent of glucose in the yolk and 0.55 per cent in the whites of the hen's egg.

#### *Testes.*

Three samples of testes were examined for glucose from the Baird salmon. The salmon were nearly mature but not one contained glucose.

<sup>8</sup> Macleod, J. J. R., *Physiol. Rev.*, 1921, i, 208.

<sup>9</sup> Tichomiroff, A., *Z. physiol. Chem.*, 1885, ix, 566.

<sup>10</sup> Henze, M., *Z. physiol. Chem.*, 1908, lv, 435.

<sup>11</sup> Kojo, K., *Z. physiol. Chem.*, 1911, lxxv, 1.

*Other Organs.*

Samples of the skin and of the stomach-intestinal mass were analyzed. The skin sample of Fish 765 from Bolinus Bay contained 0.038 per cent of glucose. Three samples of skin from spawning fish contained no glucose.

Of the three stomach-intestinal samples, two were from Monterey. One gave a trace of glucose, the other none. The specimen from Bolinus Bay contained 0.041 per cent of glucose. However, the pyloric ceca and the intestine of both Monterey and Bolinus Bay fish contained a variable quantity of mucus and unabsorbed food products. These are retained in part in the ground up total mass used for the sample, hence the 0.041 per cent of glucose from the Bolinus Bay specimen might have come from the food remnants, though it is improbable.

The stomach and intestines of McCloud River fish are greatly atrophied and small. However, no glucose analyses were obtained.

## DISCUSSION.

Kilborn and Macleod<sup>2</sup> have emphasized the comparatively low content of glycogen in invertebrates and fishes. The four fish species examined by them were dogfish, chimæra, carp, and lake trout. In their Table IV they present analyses of muscle, heart, and liver of dogfish, of carp, and of trout and give determinations for the liver of the chimæra. In the dogfish their average for body muscle is 0.018 per cent, for carp it is 0.29 per cent, and in lake trout muscle there is only a trace of glucose. Our analyses of the sea run salmon check against these determinations, 0.015 per cent in the salmon muscle in comparison with 0.018 in the dogfish and 0.29 in the carp.

On the other hand, the liver determinations of Kilborn and Macleod show as much as 5.6 per cent of glucose in the carp liver but only a maximum of 0.16 per cent for dogfish liver and 0.055 per cent for the liver of lake trout. The lake trout figure checks with that of our fasting salmon. They find a trace of glucose in lake trout muscle (probably fasting) while we find none in the fasting salmon. They have presented no analyses of the glucose content of either eggs or ovaries of fishes. In fact we have not been able to find such in the literature.



No previous comparisons have been made of the glucose content of tissues during long starvation, comparisons for which the salmon migratory habit without food lends a rare opportunity. A study of our data will show that the muscle glucose, presumably glycogen, is present in low amount during the feeding period, but drops to a trace at the beginning, and disappears entirely during the migration. Since it has been shown in several of my earlier articles<sup>1</sup> that the large store of fats progressively decreases with the migration, one can scarcely escape the view that the carbohydrates play little part in supplying kinetic energy for the migration; in fact the small amount of glucose in the muscles of feeding salmon may well be present by virtue of the digestive and anabolic processes going on at that time.

That glycogen is not entirely absent in salmon metabolism during the migratory journey is indicated by its constant presence, though in small amount, in the liver and ovaries. In view of the well known liver glycogenic function in vertebrates, and especially in mammals, it is surprising to find so small a percentage present in the salmon liver even under the most favorable conditions of feeding. In our determinations it has never exceeded 0.70 per cent of the wet weight of the organ. Livers from the Monterey feeding fish, in which the glycogen reaches its highest amount, invariably contain a considerable percentage of fat. For example, in the liver of No. 765 from Bolinus Bay unpublished analyses reveal as much as 25.8 per cent of fat, which is the highest liver fat observed. In general the normally small percentage of glycogen is greatly diminished in the spawning salmon. If we contrast the Monterey fish with the Baird spawning fish the averages for the liver are 0.405 and 0.057, respectively.

We are most pleased by the discovery of the constant composition of the ovaries in glucose (glycogen). The average for the entire series is 0.096 per cent. It seems to us to point directly to a uniform synthetic and storage process in this tissue, removed as it is from any part in energy production during the migration, but constantly growing and actively storing foods. This uniformity of composition as regards glucose adds one more point in evidence to the unpublished data of the author showing uniformity of composition of the food-loaded protoplasm of the egg cell at whatever stage of its growth it be considered.

The protein and fat percentages of fish from this series were published in 1915.<sup>7</sup> We reproduce the averages of the table in that paper but with columns for glucose introduced. Unfortunately we have no glucose determinations for tide-water fish. Both proteins and fats are stored in the mature salmon muscle in quite large excess. This storage of proteins and fats is also true for salmon ovaries.<sup>12</sup> The muscle protein excess is 6 per cent of the tissue figured on the protoplasmic basis. The fat is stored

TABLE II.

Protein, fat, and glucose in wet muscle samples giving average percentages for Monterey Bay, tide-water on the Sacramento River, and the spawning beds on the McCloud River.

Station.	Protein.*	Fat.*	Glucose.	Glucose in liver.	Glucose in ovary.
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Monterey Bay.....	15.6	18.0	0.015	0.606	0.13
Tide-water.....	16.9	14.6			
McCloud River.....	14.4	1.6	0.000	0.130	0.09

\* Protein and fat taken from Greene's<sup>7</sup> table.

to 18 and more per cent of the moist tissue (25 to 30 per cent of the dark muscle). No such large storage of carbohydrate occurs in any salmon tissue. Carbohydrate is never present in more than 0.70 per cent even in the livers of feeding salmon.

Carbohydrate is always present in the growing ovary, is in small amount in all the tissues of the feeding salmon, but disappears from the muscles and drops to a lower level in the liver during the migratory fast.

<sup>12</sup> Greene, C. W., *J. Biol. Chem.*, 1921, *xlvi*, 59.

## VITAMINE REQUIREMENTS OF CERTAIN YEASTS AND BACTERIA.

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(Received for publication, July 30, 1921.)

In a previous publication (1) we described a practical method for testing the vitamine requirements of yeast, based upon the work of Ide and his coworkers, and also of Williams, of Bachmann, and of Eddy. At that time we thought our test was specific for antiberi-beri vitamine; however, because of certain differences, the question was left open.

In this connection, Emmett and Stockholm (2) suggested that the vitamine necessary for the growth of yeast had nothing to do with antiberi-beri vitamine. Later Fulmer, Nelson, and Sherwood (3), Souza and McCollum (4), and MacDonald and McCollum (5), claimed that by improving the medium, results could be obtained similar to those noted after vitamine addition. These claims have been disproved by the work of Eddy, Heft, Stevenson, and Johnson (6), and our own findings are in accord with their conclusions in this particular.

Our present results show rather conclusively that yeast requires for growth a different substance than that needed by animals, since we were able to separate from autolyzed yeast one substance active for yeast and another for rats and pigeons. This separation will enable us to study each substance individually. At the same time, in agreement with our findings, Ide (7) shows that by improving the medium one can actually obtain a slightly better growth, not to be compared in magnitude, however, with the action of the specific vitamine-like substance.

Although the work thus far does not shed immediate light on the test for the antiberi-beri vitamine, it is of importance in the study of the vitamine requirements of yeasts and bacteria. We believe that we are dealing here with a specific substance—either

a new vitamine or a cleavage product of antiberi-beri, or vitamine B. We wish to point out that although our yeast test previously described does not specifically indicate the vitamine B activity, still it does show, to a certain extent, the relative richness in water-soluble vitamins when we are concerned with naturally occurring foodstuffs.

Simultaneous experiments with yeast and a strain of streptococcus obtained from Mueller (8), and following his method of testing, tend to show a close, if not fully established relationship between their nutritive requirements. It would be very attractive to consider these nutritive elements as one substance, but although there are many points in common, there are still many differences to be reconciled, so that the question must be left open for the present.<sup>1</sup>

Another thing worthy of note is that different strains of yeast behave differently as regards vitamine requirement. Some of them, as shown by Nelson, Fulmer, and Cessna (9), seem to be able to synthesize their own vitamine, the initial inoculation providing the first impulse, while others require the addition of extra vitamine.<sup>2</sup>

This difference in the vitamine requirements of various strains of yeast may shed some light on the ultimate physiology of yeast cells. These lower organisms having greater synthetic power appear to be able to utilize the simplest type of vitamine, so that chemically it might be advantageous to study the structure of vitamins in this way.

#### EXPERIMENTAL.

*Differentiation between the Substance Active for Yeast and That Active for Pigeons and Rats.*—In our previous paper, we showed that much larger quantities of fullers' earth were necessary to remove from autolyzed yeast the substance necessary for the

<sup>1</sup> Detailed experiments along this line are being conducted in our laboratory by L. Freedman, and the results will be presented in a later publication.

<sup>2</sup> Peters (10) believed at first that protozoa can live and divide on purely inorganic material, but he found subsequently that they became smaller and smaller and appeared to live at the expense of their own protoplasm; he thinks, therefore, that addition of vitamins is necessary for proper growth.

growth of yeast, than those which are known to be sufficient for the removal of vitamine B; this is in agreement with the findings of Emmett and Stockholm (2). Our present results show that the substance active for yeast may be removed almost quantitatively from autolyzed yeast by two successive shakings each with

TABLE I.

No.	Substance tested.*	Net yeast activity.	Animal activity.
		<i>mm.</i>	
1	Autolyzed yeast.....	14.5	Positive.
2	“ “ shaken with fullers' earth (50 gm. per liter).....	12.0	Negative.
2a	Fullers' earth from No. 2 decomposed with baryta.....	4.0	Positive.
3	Autolyzed yeast (filtrate from No. 2) shaken with fullers' earth (100 gm. per liter).....	6.0	Negative.
3a	Fullers' earth from No. 3 decomposed with baryta.....	3.5	“
4	Autolyzed yeast (filtrate from No. 3) shaken with fullers' earth (100 gm. per liter).....	0.5	“
4a	Fullers' earth from No. 4 decomposed with baryta.....	0	“
5	Autolyzed yeast shaken with norit (50 gm. per liter).....	13.5	“
5a	Norit from No. 5 decomposed with glacial acetic acid.....	3.0	Positive.
6	Autolyzed yeast (filtrate from No. 5) shaken with norit (100 gm. per liter).....	3.0	Negative.
6a	Norit from No. 6 decomposed with glacial acetic acid.....	3.0	“
7	Autolyzed yeast (filtrate from No. 6) shaken with norit (100 gm. per liter).....	0.5	“
7a	Norit from No. 7 decomposed with glacial acetic acid.....	0	“

\* In each case the amount tested was 0.05 cc., so that the results are quite comparable.

100 gm. of fullers' earth or of norit, per liter. It is essential that with every lot of autolyzed yeast, controls must be run to determine the degree of separation. The fullers' earth and norit were decomposed with baryta and glacial acetic acid respectively, according to the method of Seidell, and of Eddy and coworkers. Norit, extracted with baryta, did not yield any active substance.

The various fractions were also tested on rats and pigeons with concordant results, both preventive and curative experiments being performed.

*Specificity.*—The following experiment shows that by improving the medium either in its inorganic or organic moiety (glucose, proteins) the growth of yeast cells can sometimes be improved; however, the magnitude of the resulting response is of an entirely different order than that obtained by vitamine addition. Using the inorganic medium of Fulmer, Nelson, and Sherwood (Medium F) we did not obtain any more growth than on our Nägeli solution. Using Medium F and autolyzed yeast we had even lower results than with Nägeli solution. This corroborates the finding of Eddy, Heft, Stevenson, and Johnson (6).

No.	Medium.	Yeast activity.	Net activity.
		mm.	mm.
1	Blank determination (Medium F).....	3.0	
2	“ “ (Nägeli).....	3.0	
3	Medium F plus 0.05 cc. of autolyzed yeast plus yeast suspension.....	12.5	9.5
4	Nägeli plus 0.05 cc. of autolyzed yeast plus yeast suspension.....	14.0	11.0
5	Medium F plus 0.05 cc. of autolyzed yeast.....	0	
6	Nägeli plus 0.05 cc. of autolyzed yeast.....	0	

The results with glucose have shown that an addition of the sugar has little or no effect, contrary to the findings of MacDonald and McCollum (5). It seems that the slight effect obtained with glucose can be eliminated by shaking the sugar solution with an adsorbent.

Ground up meat was extracted and autoclaved till a watery extract no longer showed any vitamine activity. The meat was then subjected to hydrochloric acid hydrolysis, neutralized, and tested again. There was a small but definite activity manifested. The same was true of casein and gelatin but not of zein, egg albumin, or serum albumin. These experiments are being continued.

The addition of glucose to the blank determination does not affect the result.

No.	Medium.	Yeast activity.	Net activity.
		mm.	mm.
1	0.05 cc. of autolyzed yeast.....	12.5	10.0
2	0.05 cc. of autolyzed yeast plus 1 cc. of 10 per cent glucose.....	13.5	11.0
3	0.05 cc. of autolyzed yeast plus 1 cc. of 10 per cent glucose shaken with 10 per cent charcoal (norit). ....	13.5	11.0
4	0.05 cc. of autolyzed yeast plus 1 cc. of 10 per cent glucose shaken with 10 per cent fullers' earth.....	12.5	10.0
5	0.05 cc. of autolyzed yeast plus 1 cc. of 10 per cent glucose shaken with 10 per cent Lloyd's reagent.....	12.5	10.0
6	Blank (without autolyzed yeast).....	2.5	
7	1 cc. of 10 per cent glucose.....	2.5	0

Regarding the activity of bakers' and brewers' yeast we have found, in agreement with Williams (11) that brewers' yeast extract is more potent than bakers' yeast extract in affecting the growth of brewers' yeast. On the other hand, contrary to Williams (11) we have noted that bakers' yeast extract does not stimulate the growth of bakers' yeast as much as does brewers' yeast extract.

Goy (12) claims to have isolated a nitrogen-free acid which stimulates the growth of bacteria and yeast, but a closer examination of his results does not lend support to his claims, the substance actually isolated being entirely inactive.

Preliminary work shows that there are many points of similarity and dissimilarity between the substance stimulating the growth of yeast and that stimulating the growth of streptococcus. We have found for example that undecolorized heart infusion exhibits a very marked yeast growth-stimulating activity, while the decolorized infusion (decolorized with norit) shows only a very negligible activity. Autolyzed brewers' yeast also showed marked stimulating action on the growth of streptococcus.

Peptone added to the medium gives growth both with yeast and with streptococcus. Casein hydrolysate acts slightly on yeast growth and more so on streptococcus. Hydrolysates of some other proteins did not act on yeast but were active for

streptococcus. It is just such discrepancies as these that make it imperative to obtain more data before reaching definite conclusions. The method of separation from vitamine B of the substance stimulating yeast growth, and which we will provisionally call "vitamine D," will facilitate further work and may help to clear up the question of the identity of vitamine D with the substance stimulating the growth of streptococcus.

#### CONCLUSIONS.

We have separated from vitamine B a substance which we shall call provisionally vitamine D and which acts on microorganisms.

Vitamine D appears to be a definite and specific substance stimulating the growth of yeast.

Streptococcus is more difficult to study because apparently it needs at least two substances for growth.

Although vitamine D has been obtained free from vitamine B, as far as our animal experiments have shown, the reverse is not true. It is evident, therefore, that most animal tests conducted up to the present were carried out with a mixture of vitamines B and D and will consequently have to be repeated as soon as a clear separation of the two substances can be effected. It may develop that the vitamine D, obtained from yeast, and the vitamine-like substance obtained from proteins, such as casein, may have some special function in the body, and such experiments are now being planned.

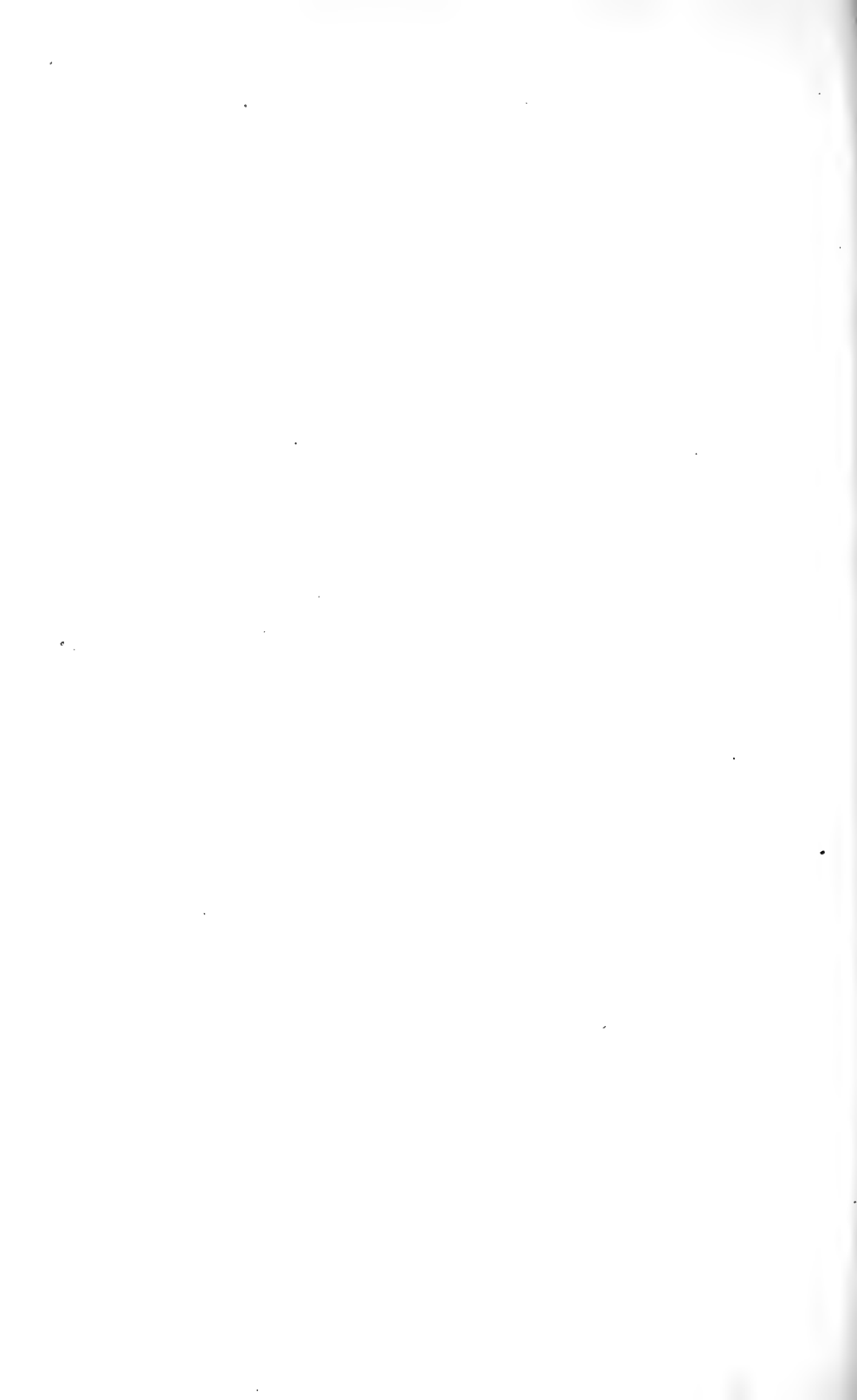
Regarding the possible identity of the substance promoting the growth of yeast with that influencing the growth of streptococcus, our present data are insufficient to venture a definite statement.

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## THE EFFECT OF SUBCUTANEOUS INJECTIONS OF SOLUTIONS OF POTASSIUM CYANIDE ON THE CATALASE CONTENT OF THE BLOOD.

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(Received for publication, June 20, 1921.)

In 1889 Geppert<sup>1</sup> concluded on the basis of an extensive series of experiments that potassium cyanide acted on the living organism through the mechanism of making the cells lose their power of oxygen utilization. He states that the picture is one of internal suffocation in the presence of excess oxygen. His experimental results showed that the oxygen consumption was very markedly diminished, and also that the carbon dioxide formed was very markedly diminished. Scientific investigators seem to have accepted Geppert's results and interpretation as final, since no experiments have been carried out since that time along precisely the same lines.

In recent years a theory has been built up to the effect that catalase of the blood (by which we understand the catalytic activity which greatly accelerates the breaking down of hydrogen peroxide) follows the oxidative capacity. Further, that anything which affects the catalase content of the blood must necessarily have a similar effect on the oxidative process in the organism. It appeared to us that potassium cyanide would be a satisfactory substance to apply, in testing out this theory.

The methods that had been used for the estimation of catalase at the time this work was planned, appeared to be rather crude, and so an apparatus was built in which the liberated gas could be measured under conditions corrected for pressure and where the shaking could be done mechanically under constant conditions.

<sup>1</sup> Geppert, J., *Z. klin. Med.*, 1889, xv, 307.

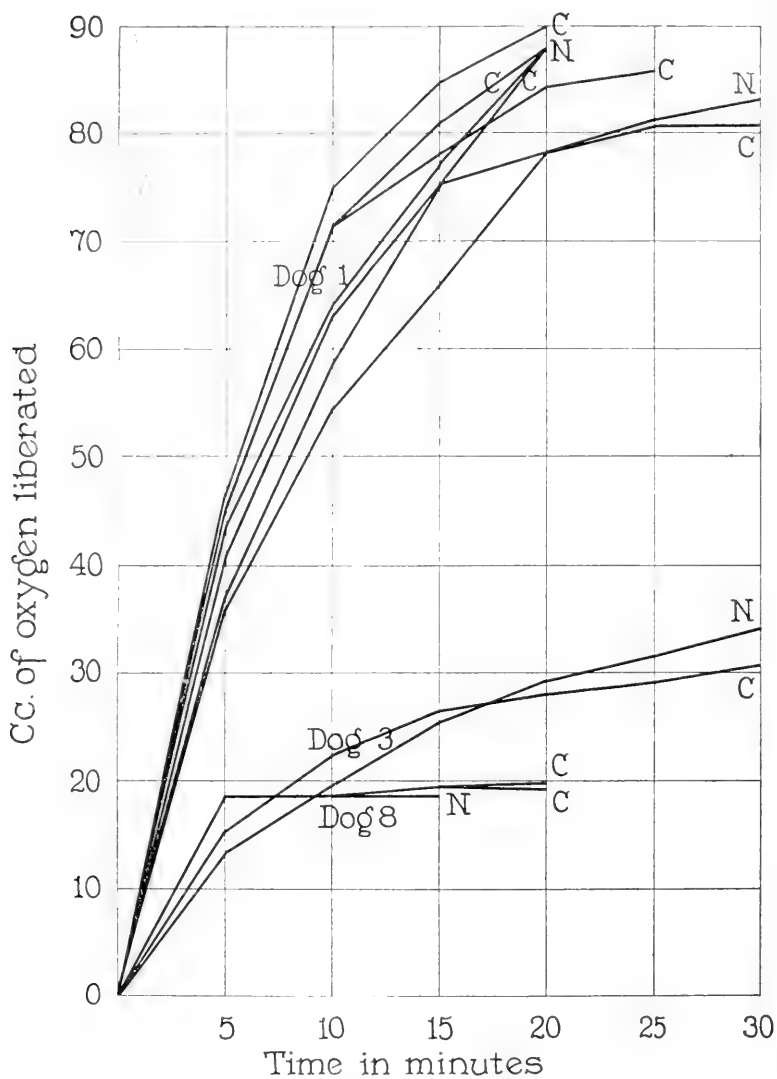


FIG. 1.

A description<sup>2</sup> of this apparatus is being published in another journal. Our determinations were carried out in triplicate and practically all the results given are the averages of closely agreed triplicates. In all but one of our series of experiments, samples of 0.5 cc. of oxalated blood were used. The exception was in the case of Dog 8 in Fig. 1, in which samples of 0.25 cc. were used. The sample was placed into a small aluminium vessel and floated on top of 75 cc. of equal volumes of hydrogen peroxide and distilled water. The stoppers were then carefully placed into the bottles, the gas levels adjusted, the stop-cocks on the bottles closed, and the machine was started. The volumes of gas, liberated, were read at 5 minute intervals, run off by a timer, and the shaking was continued until the curves became practically flat. From an examination of the curves obtained from normal bloods (Fig. 2) it is apparent that final readings taken at the end of a 10 minute period do not give results that can be satisfactorily compared. This 10 minute period has been used by a number of investigators in the estimation of catalase. These curves also show that there is quite a marked variation between different normal bloods. In these experiments potassium cyanide was injected subcutaneously in such amounts as to produce death. The blood samples were removed from the femoral artery by means of a cannula. A small quantity of powdered potassium oxalate was used to prevent clotting. The starred curve for Dog 4 in Fig. 3 was obtained from blood mixed with potassium cyanide *in vitro* in the same proportion as was injected. From an examination of the curves from our eight experiments (Figs. 1, 3, 4) it becomes apparent that there is a diminution in the catalase content of the blood in only one case. In these figures the curves marked N were obtained from normal blood and those marked C from blood after injection of potassium cyanide. This is true even when the blood was taken during coma, and in two cases where the blood was tested after death.

From the experimental results we conclude that lethal doses of potassium cyanide injected subcutaneously have practically no effect on the catalase content of the blood; secondly, that if the generally accepted theory of Geppert as to the mode of action of

<sup>2</sup> Welker, W. H., *J. Lab. and Clin. Med.*, 1921, vii, in press.

potassium cyanide is correct, there can be no connection between oxidase and catalase.

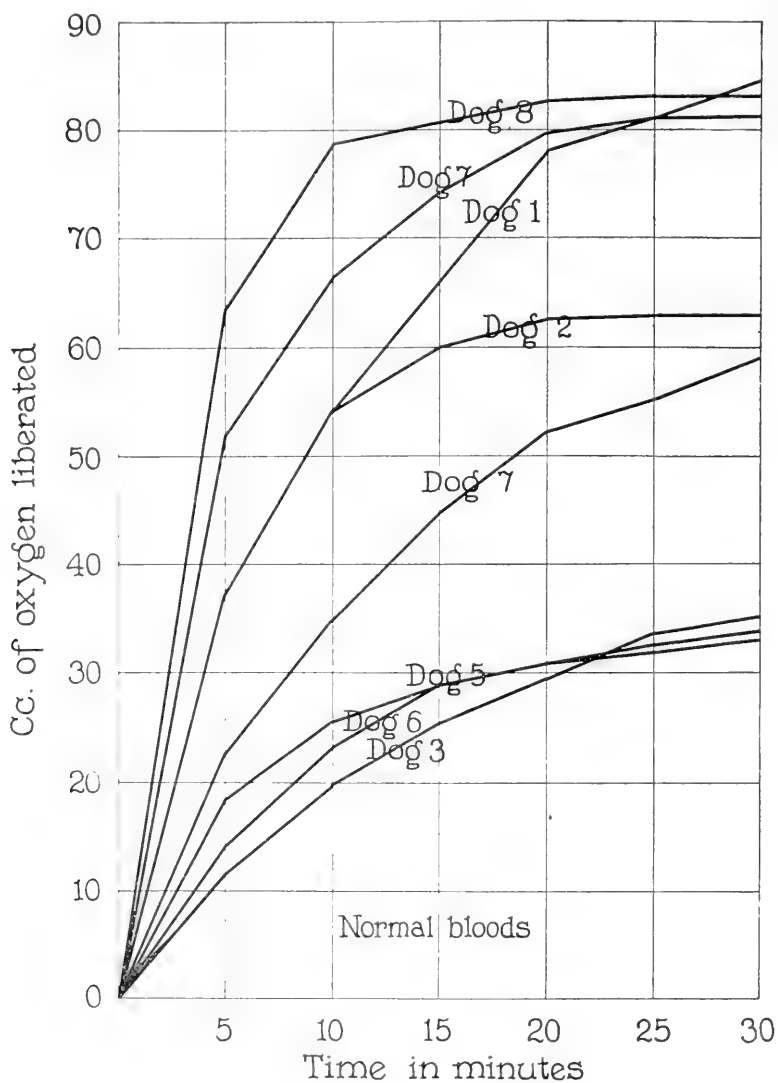


FIG. 2.

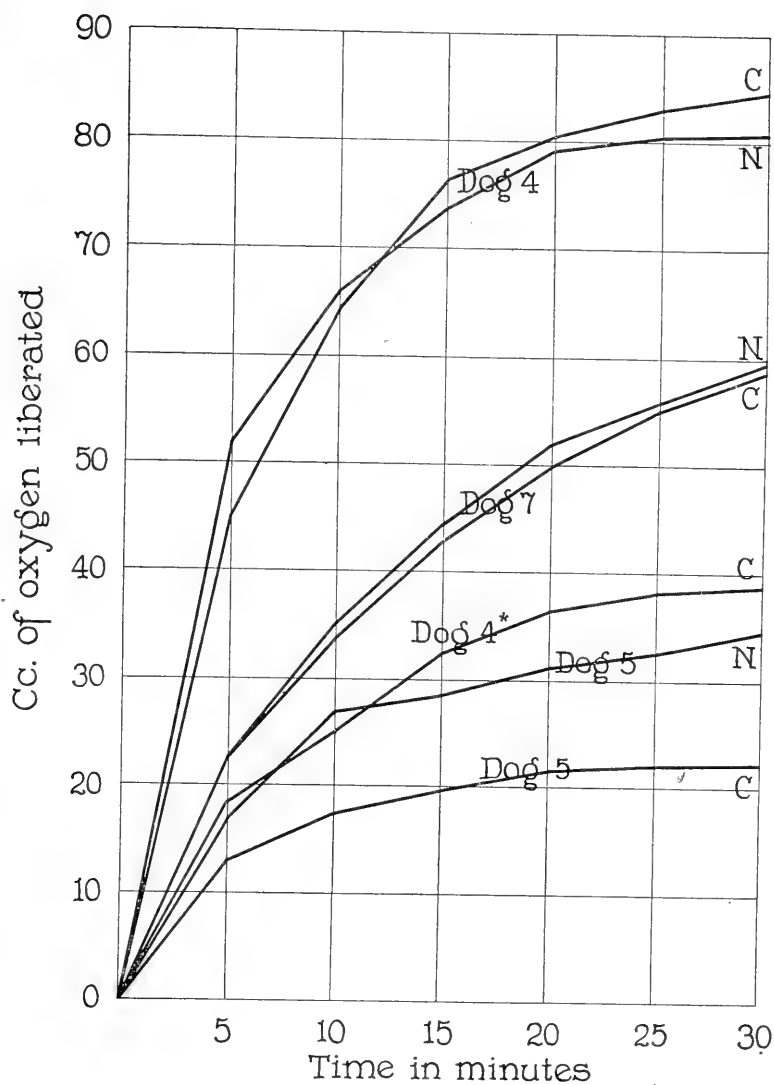


FIG. 3.

*Protocols.*

*Dog 1.*—Two samples of normal blood were taken at an interval of 15 minutes. 3 mg. per kilo of 5 per cent KCN solution injected at 11.50 a.m. Second injection of same dose at 12.35 p.m. Blood sample taken at 12.40 p.m. 6 mg. per kilo of 5 per cent KCN solution injected at 12.55 p.m.

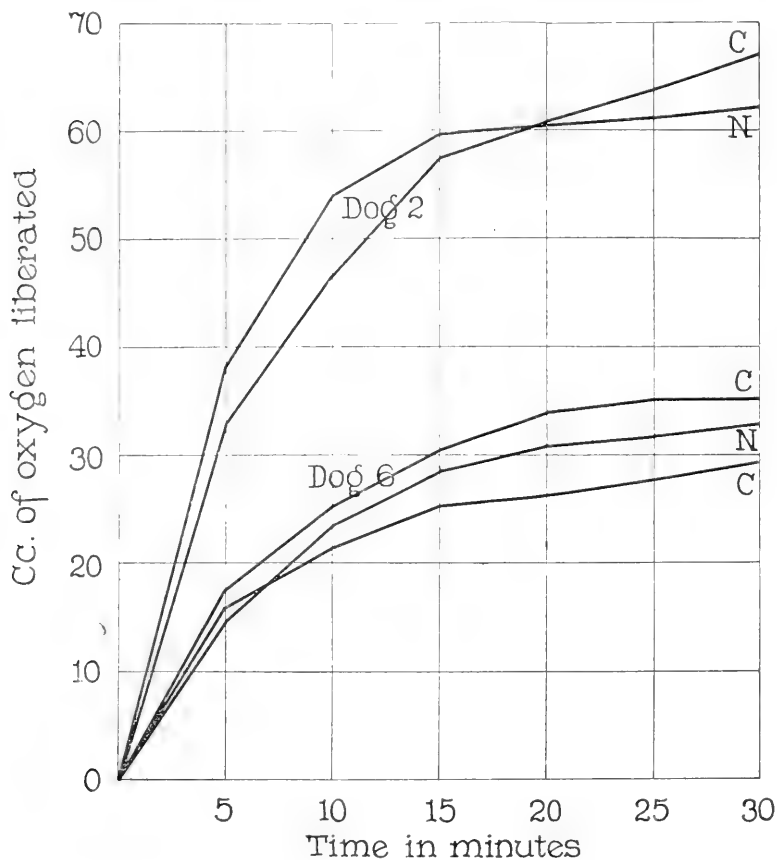


FIG. 4.

Blood sample taken at 1.05 p.m. Blood sample taken at 1.55 p.m. The animal was in convulsions at this point. Death occurred at 2.00 p.m. Another sample of blood was taken at this time.

*Dog 2.*—Blood taken for normal sample. 8 mg. per kilo of 5 per cent KCN solution injected at 1.20 p.m. Injection repeated at 1.30 p.m. Death occurred at 1.45 p.m. Blood removed from the heart at 1.48 p.m.



*Dog 3.*—Blood taken for normal sample. 10 mg. per kilo of 5 per cent KCN solution injected at 12.35 p.m. Death occurred at 1.00 p.m. Sample of blood removed from the heart within 30 seconds after the occurrence of death.

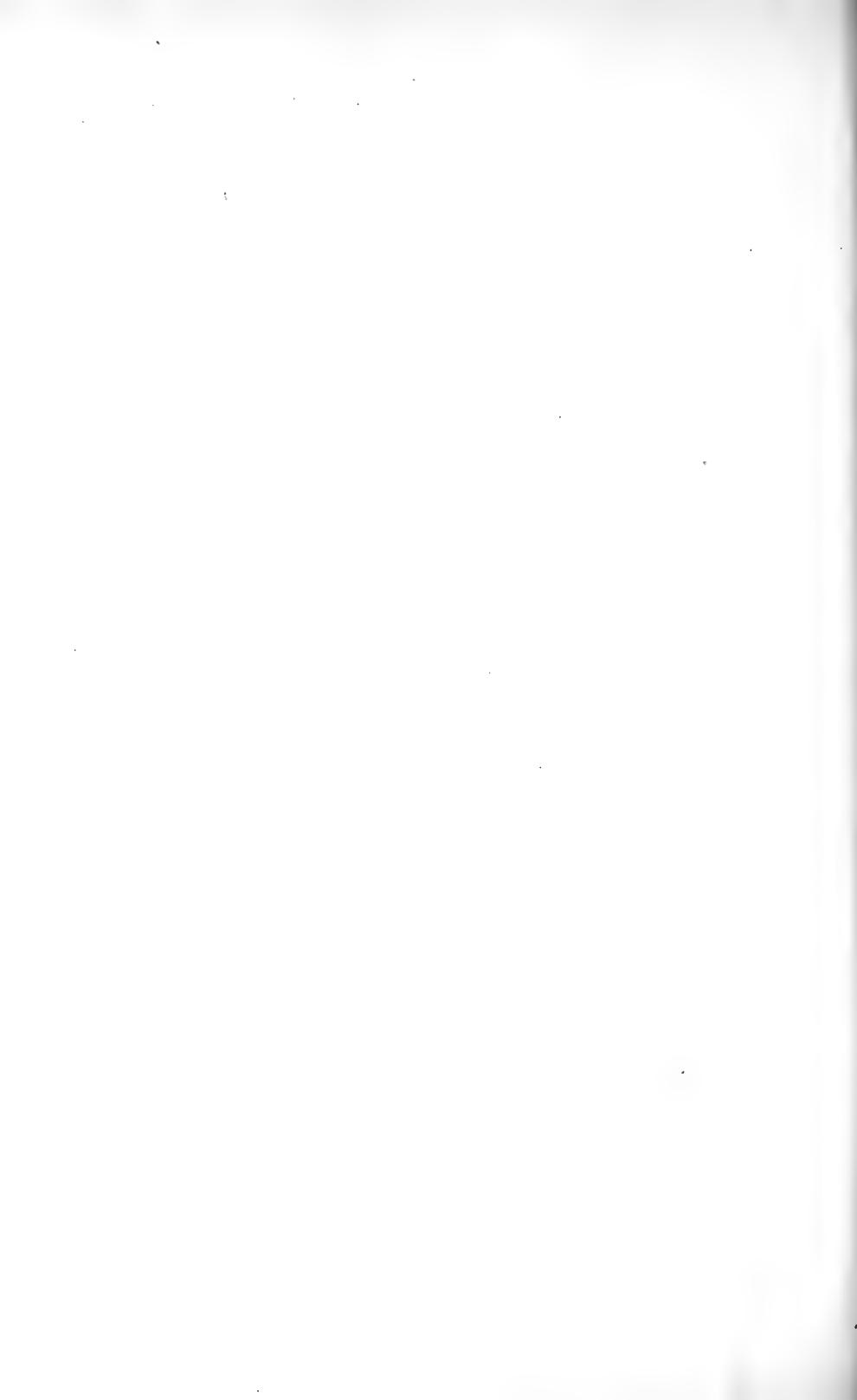
*Dog 4.*—Blood taken for normal sample. 8 mg. per kilo of 5 per cent KCN solution injected at 2.15 p.m. Injection repeated at 2.30 p.m. Blood sample taken at 2.40 p.m. Death occurred at 2.50 p.m.

*Dog 5.*—Blood taken for normal sample. 10 mg. per kilo of 5 per cent KCN solution injected at 11.40 a.m. Convulsions at 11.43 a.m. Blood taken at 12 noon. Death occurred at 12.20 p.m.

*Dog 6.*—Blood taken for normal sample. 8 mg. per kilo of 5 per cent KCN solution injected at 11.11 a.m. Convulsions and labored breathing at 11.25 a.m. Blood sample removed at 11.38 a.m. and 1.55 p.m. Death occurred at 2.20 p.m.

*Dog 7.*—Blood taken for normal sample. 8 mg. per kilo of 5 per cent KCN solution injected at 10.15 a.m. Blood removed at 11.05 a.m. The excitement stage had reached its maximum at this point. Death occurred at 2.00 p.m.

*Dog 8.*—Blood taken for normal sample. 10 mg. per kilo of 5 per cent KCN solution injected at 4.05 p.m. 6 mg. per kilo of 5 per cent KCN solution injected at 4.30 p.m. Marked convulsions at 4.37 p.m. Blood sample taken at the point of death at 4.45 p.m.



## CITRIC ACID CONTENT OF MILK AND MILK PRODUCTS.

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(Received for publication, July 23, 1921.)

Citric acid has been recognized as one of the normal constituents of milk for many years, but there is still some disagreement as to the forms in which it exists in this product. Soldner (1) claims the presence of potassium, magnesium, and calcium citrates, whereas Van Slyke and Bosworth (2) state that only sodium and potassium salts of this acid are present. Regardless of the exact form in which this constituent is found in milk, quantitative determinations have shown that there is normally between 0.1 and 0.2 per cent citric acid combined in one form or another.

Interest has been directed to the parallelism between the citric acid content of fruit juices and their antiscorbutic properties, and to the similar association of these factors in milk. If only natural products, unheated and without subjection to processing for preservation, were considered, the existence of a definite relationship between the two factors would be more acceptable. Reliable experimental evidence, however, shows that the antiscorbutic properties of natural foods are destroyed by heat whereas the citric acid content of the same products is not quantitatively affected by the application of heat even in excess of that required for the destruction of the antiscorbutic property. Sommer and Hart (3) have shown that milk may be autoclaved at 15 pounds pressure for 1 hour without causing a diminution in its citric acid content. They have also shown that the citrates of milk are not rendered insoluble by autoclaving for 20 minutes at 15 pounds pressure.

Since comparatively recent investigations by Hess (4, 5), Hart (6, 7), and Dutcher (8) and their coworkers have made available additional data on the antiscorbutic potency of milk as affected

by the feed of the lactating animal, and by what appears to be the method of processing in the preparation of various concentrated milk products, it has been considered desirable to record the variations in citric acid content of milk from individual cows while on a normal winter ration and when on a normal summer or pasture ration; also to determine the citric acid content of some well known concentrated milk products.

#### EXPERIMENTAL.

##### *Estimation of Citric Acid in Milk.*

Due to the small amount of citric acid present in milk and because of the possible variations which it was desirable to detect, it has been deemed advisable to incorporate details of the well known methods used in this series of analyses.

*Determination of Citric Acid in Milk.*—50 cc. of milk are treated with 10 cc. of dilute sulfuric acid (1:1) and thoroughly agitated. 2 cc. of 40 per cent potassium bromide solution and 20 cc. of a solution of phosphotungstic acid are then added. After a thorough mixing, the precipitate is separated by filtration. To the perfectly clear filtrate in an Erlenmeyer flask is added an excess of freshly prepared saturated bromine water (usually between 5 and 10 cc.). The mixture is then placed on the water bath at a temperature of from 48-50°C. for about 5 minutes. After removing from the bath, add rapidly from a burette 25 cc. of potassium permanganate solution (5 per cent) drop by drop with frequent interruptions, and with constant and vigorous shaking, avoiding a temperature during the oxidation exceeding 55°C. Set the flask aside until the hydrated peroxide of manganese begins to settle. The supernatant liquid should be dark brown showing an excess of permanganate. Add more permanganate if an excess is not indicated. When the precipitation assumes a yellow color and most of it is dissolved, add drop by drop a clear solution of ferrous sulfate until the hydrated peroxide of manganese and excess of bromine are removed. Allow the solution to cool, shaking occasionally. Allow the mixture to stand over night. Collect by means of gentle suction on a tared Gooch crucible provided with a thin pad of asbestos previously dried over sulfuric in a vacuum desiccator; wash with water slightly acidified with sulfuric acid and finally wash twice with water. Dry the precipitate to constant weight over sulfuric acid in a vacuum desiccator protecting the precipitate from strong light. The weight of the precipitate multiplied by the factor 0.424 gives the equivalent weight of anhydrous citric acid in the sample.

*Determination of Citric Acid in Milk Powder.*—Weigh 5 gm. of powder into a beaker and reconstitute with 45 cc. of warm water. Mix thoroughly and proceed as with liquid milk.

*Determination of Citric Acid in Sweetened Condensed Milk.*—Weigh out 25 gm. of the sample and add 200 cc. of 95 per cent alcohol. Mix thoroughly and filter. To the filtrate add enough 0.25 N barium hydroxide to almost neutralize the solution and then 5 cc. of 50 per cent barium acetate in order to insure an excess of barium. Add about 150 cc. of 95 per cent alcohol and reflux until the precipitate settles readily after being shaken. Filter and thoroughly wash the precipitate in the flask and on the paper with 95 per cent alcohol. Transfer the precipitate from the filter to the flask with a jet of hot water. Boil until alcohol can no longer be detected by odor and add enough sulfuric acid (1:5) to precipitate all of the barium originally present and to allow 2 cc. in excess. Evaporate to a volume of 60 or 70 cc.; cool and add an excess of bromine water. Filter and add 10 cc. of potassium bromide, then place on the water bath at a temperature of 48–50° C. and proceed as with liquid milk.

TABLE I.

*Percentage of Citric Acid Recovered from Milk Products.*

	Liquid milk.		Liquid milk and sugar.		Evaporated milk.		Condensed milk.	
	No. 1	No. 2	No. 1	No. 2	No. 1	No. 2	No. 1	No. 2
Original.....	0.132	0.129	0.131	0.130	0.202	0.204	0.096	0.090
After adding 0.02 per cent ..							0.110	0.104
“ “ 0.05 “ “	0.179	0.180	0.182	0.179	0.252	0.253	0.143	0.148
“ “ 0.10 “ “							0.190	0.197
“ “ 0.15 “ “	0.279	0.279	0.279	0.275				

The relative accuracy of these methods is shown in Table I in which is given the results of duplicate determinations on liquid milk with and without sugar, on evaporated milk, and on sweetened condensed milk; also duplicate results from each of these products after known amounts of citric acid in the form of sodium citrate had been added. It will be noted that the maximum variation in duplicate results does not exceed 0.006 per cent; it is believed therefore, that any significant variations occurring in the products examined were easily detected by the methods used.

*Citric Acid in Milk as Affected by Feed.*

In view of the work reported by Hess, Unger, and Supplee (4) in which it was shown that the milk produced from a highly concentrated ration contained less citric acid than that produced during pasture feeding, it has seemed desirable to obtain further

data on the amount of this constituent found in the milk of the same herd while receiving a normal winter ration and again during pasture feeding. Accordingly, samples were analyzed late in February and again late in June; the results are shown in Table II.

From the results in Table II it is evident that there is a wide variation shown in the milk from individual animals receiving the same feed as represented by the difference between 0.121 and 0.182 per cent (Herd I). While the evidence pointing toward a variation

TABLE II.

*Citric Acid Content of Milk from Winter Ration and from Pasture Feeding.*

Herd.	Cow.	Winter ration.		Summer pasture.		
		Citric acid.	Feed.	Citric acid	Feed.	
		<i>per cent</i>		<i>per cent</i>		
I	1	0.173	Hay, distiller's grains, ensilage, corn stover, molasses.	0.174	Fresh grass only.	
	2	0.121		0.114	"	"
	3	0.182		0.156	"	"
II	1	0.145	Hay straw, cottonseed, meal.		"	"
	2	0.155		0.148	"	"
	3	0.106		0.130	"	"
III	1	0.139	Hay, oil meal, corn-meal, bran.	0.164	"	"
	2	0.119		0.138	"	"
	3	0.139		0.160	"	"

in citric acid content as affected by the different feeds is not conclusive, there is, nevertheless, a tendency toward a higher percentage of this constituent in the winter milk of cows receiving ensilage and corn stover than in the milk of those herds receiving only hay as roughage. When summer and winter milks from each herd are compared there is a significant difference only in the case of Herd III in which the milk from pastured cows contains a uniformly higher citric acid content. The average citric acid content of the milk from all cows on a winter ration was 0.142 per cent and from all cows when on pasture was 0.148 per cent.

*Citric Acid Content of Concentrated Milk Products.*

Since one of the purposes of this paper is to furnish analytical data showing the citric acid content of concentrated milk products, it is desirable to briefly mention that in the manufacture of condensed, evaporated, and desiccated milks heat is applied in amounts varying from 110–112°C. for a few seconds in the manufacture of powdered milk by the Just process, to sterilization under steam pressure in the case of evaporated milk. Therefore, the results from the products analyzed will adequately cover the temperature range to which concentrated milk products are subjected during process of manufacture.

TABLE III.

*Citric Acid Content of Condensed and Evaporated Milks.*

Sample.	Milk.	Citric acid in product.	Citric acid calculated to liquid milk basis.
		<i>per cent</i>	<i>per cent</i>
1	Evaporated.....	0.168	0.084
2	“.....	0.302	0.151
3	“.....	0.295	0.147
4	“.....	0.211	0.105
5	“.....	0.255	0.127
6	“.....	0.203	0.101
7	Sweetened condensed.....	0.094	0.078
8	“ “.....	0.124	0.103

The citric acid content of six different brands of evaporated milk and two brands of sweetened condensed milk are shown in Table III; also included in this table is the citric acid content calculated to the original liquid milk basis assuming a concentration ratio of 2 to 1.

The citric acid content of milk powder made by the spray process is shown in Table IV. The concentration ratio used for calculating to the liquid milk basis is 1 to 8.5 and 1 to 12 respectively for whole milk and skimmed milk.

It has been possible to check up very closely on the citric acid content of powder made by the Just process by determining the citric acid and total solids before drying, for comparison with the dried product and with the reconstituted milk correctly diluted

on the basis of the data obtained from the total solids determinations. The results of these determinations are shown in Table V.

The results from the different concentrated and desiccated milks do not show any variation in citric acid content which could

TABLE IV.  
*Citric Acid Content of Milk Powder (Spray Process).*

Sample.	Milk.	Citric acid in product.	Citric acid calculated to liquid milk basis.
		<i>per cent</i>	<i>per cent</i>
1	Whole milk powder.....	1.26	0.148
2	“ “ “ .....	1.22	0.143
3	“ “ “ .....	1.23	0.144
4	Skimmed milk powder.....	1.70	0.141
5	“ “ “ .....	1.50	0.125
6	“ “ “ .....	1.45	0.121

TABLE V.  
*Citric Acid Content of Milk Powder (Just Process).*

Sample.	Milk.	Citric acid in milk before drying.	Citric acid in powder.	Citric acid in accu- rately re- constituted powder.
		<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
1	Skimmed milk.....	0.139	1.37	0.135
2	“ “ .....	0.105	1.05	0.104
3	“ “ .....	0.165	1.90	0.166
4	Part skimmed milk.....	0.138	1.29	0.138
5	“ “ “ .....	0.110	1.05	0.110
6	“ “ “ .....	0.170	1.76	0.167
7	Whole milk.....	0.141	1.15	0.141
8	“ “ .....	0.103	0.776	0.102
9	“ “ .....	0.173	1.45	0.177

not possibly be explained by variations in the liquid milk prior to manufacture. In the case of the Just process powders where complete data were available for reconstituting powder to the original milk basis, the citric acid content before and after drying is in full agreement. These results are therefore in full accord



with those of Sommer and Hart (3) and others in that the heating of milk does not cause a decrease in its citric acid content. The results also serve as further evidence to the lack of a definite relationship between citric acid content and antiscorbutic potency, as there is experimental evidence showing that some of these concentrated milk products apparently lack this vitamine, whereas in others it is retained to a high degree.

*Citric Acid Content of Milk as Affected by Age and Bacterial Quality.*

From the preceding data it has appeared that the variations in citric acid content of concentrated milk products could be explained

TABLE VI.  
*Citric Acid Content of Milk as Affected by Aging.*

Sample.	Age.	Acidity.	Citric acid.
		<i>per cent</i>	<i>per cent</i>
Raw.....	Fresh.	0.182	0.134
" .....	3 days.	0.250	0.129
" .....	4 "	0.500	0.121
" .....	14 "	0.550	0.104
" .....	28 "	0.650	0.042
Pasteurized.....	Fresh.	0.160	0.134
" .....	7 days.	0.160	0.134
" .....	14 "	0.180	0.134
" .....	28 "	0.550	0.087

by the variations in the milk from individual animals rather than by the method of manufacture. Another possible cause of variation is suggested, however, by the observations of Kunz (9) to the effect that citric acid gradually decreases with the aging of milk, particularly when soured. The utilization of citrates by certain microorganisms is an established fact and it would seem quite possible that under certain conditions and in the presence of certain species of bacteria, the citric acid content of milk might be appreciably lowered. In order to secure information on this point, a sample of milk held raw and after pasteurization for a period of 28 days was analyzed for developed acidity and citric acid content at regular intervals. The samples were held at low

temperatures after the first 3 days. The results of these determinations are shown in Table VI.

Even though the results given in Table VI show a marked decrease in citric acid content due to aging, there is considerable doubt as to just how far these results can be used as an explanation of the variations recorded in the preceding tables, particularly in view of the knowledge that milk for manufacturing purposes must of necessity be fresher and of better quality than that in which the diminution in citric acid was detected. The results, however, are significant as indicating the general tendency toward reduction in citric acid content in milk of poor quality.

#### SUMMARY.

The conclusions which may be drawn from these investigations follow.

There is a marked variation in citric acid content of the milk from individual animals, which may be explained on the basis of the data shown herein as due to the individuality of the particular animal. Certain data, however, indicate that the ration may have a slight effect upon this constituent.

There is apparently no effect upon the citric acid content of milk caused by heating during the manufacture of evaporated, condensed, and dried milks. The results indicate that the amount found in each of these products, if subject to variation, must be attributed to causes other than heat.

The parallelism between citric acid content and antiscorbutic properties does not hold true in the case of concentrated milk products; the potency of this factor has been shown to be absent in some of the heated products and present in others. The citric acid content however, seems to be present in all of them apparently to the same degree as found in natural raw milk.

The citric acid content of milk decreases during aging in the presence of high developed acidity, and is more rapid in raw milk than in pasteurized milk. It is quite probable that the effect of acidity and age is not applicable in causing as marked a diminution of citric acid in milk used for manufactured products as is shown by the data presented herein.

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## THE AMMONIA CONTENT OF THE BLOOD, AND ITS BEARING ON THE MECHANISM OF ACID NEUTRALIZATION IN THE ANIMAL ORGANISM.

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(Received for publication, August 11, 1921.)

A review of the very full literature upon the concentration of ammonia in blood discloses much confusion and divergence in the reported values. For example, the range of values for the systemic blood of normal dogs is from a few hundredths of a mg. (Folin and Denis, 1) to 5 mg. (MacCallum and Voegtlin, 2) or more of ammonia nitrogen in 100 cc. Even more pronounced are variations in the data of comparable pathological states; yet in many instances the blood ammonia has been determined purely incidental to the investigation of problems of intermediary metabolism, and made the basis for hypotheses whose validity depends upon not only the relative but absolute accuracy of the analyses. With improvements in technique, and a growing appreciation of the inherent difficulties in the estimation of ammonia in blood there has been a preponderant (but by no means uniform) trend to the lower extreme of values, until, according to Myers (3), "there appears to be some question at the present time as to whether ammonia actually exists in the blood."

In 1896, Nencki and Zaleski (4) adapted to the estimation of ammonia in blood the vacuum distillation procedure originally proposed by Boussingault (5) and more recently employed by Wurster (6). 50 to 100 gm. of blood and an equal volume of filtered lime water were distilled for 3 hours, at a pressure of 10 to 15 mm. and temperature not exceeding 35°C. The ammonia was received in 0.1 N sulfuric acid and the excess acid titrated by 0.025 N sodium hydroxide solution, with methyl orange indicator. By this method Nencki and Zaleski found in 100 gm. of dog blood the following values: arterial blood, from 1.4 to 2.8 mg. of ammonia; portal blood, 5.2 mg.; and hepatic venous blood, 1.9 mg. They realized, however, that

higher temperatures, longer distillation, or greater concentration of alkali, would give higher values.

This method found immediate application in the very interesting series of experiments which were then being carried out by the Nencki-Pawlow school on dogs with Eck fistula, with the view of determining the site of urea formation in the animal body. Various experimental evidence had accumulated indicating that urea formation was a function of the liver. Thus, Minkowski (7) had shown that after extirpation of the liver in geese there is a very great fall in uric acid excretion and a corresponding increase in ammonia excretion. Von Schröder (8) had disposed of the old notion that the kidneys were able to form urea, and had found that perfusion of the liver with blood containing ammonium carbonate produced an increase in the urea content of the blood. Furthermore, it had been observed that Eck fistula dogs show symptoms of acute poisoning on a heavy meat diet. Nencki, Pawlow, and Zaleski (9), Nencki and Pawlow (10), Salaskin (11), Salaskin and Zaleski (12), and others, employing the new vacuum distillation method, found that the blood of the portal system contains three to four times as much ammonia as arterial blood; that after Eck fistula operation in dogs, with extirpation of the liver or ligation of the hepatic arteries, the ammonia content of arterial blood and the excretion of ammonia are increased; that during the acute poisoning of meat-fed Eck fistula dogs the ammonia content of the arterial blood approaches the values found for portal blood. The conclusion seemed well established that the chief site of urea formation, in mammals, at least, is the liver, and that the ammonia split off from protein during intestinal digestion is converted into urea in the liver.

The experimental findings and conclusions of the Petersburg school did not go unchallenged even at this early date. Biedl and Winterberg (13) were unable to find a constant difference between the ammonia of arterial and portal blood, and stated that the ammonia value is directly dependent, within wide limits, on the ratio between the amounts of blood and lime water employed in the Nencki-Zaleski procedure. Nencki and Zaleski (14) confirmed this criticism of their original method and introduced several modifications including the use of magnesium oxide instead of lime water, and distillation at a lower temperature and for a shorter time. With the method thus modified they found as the average value for arterial blood of fifteen dogs 0.35 mg. of ammonia, and for portal blood 1.45 mg. of ammonia, in 100 gm. of blood. They also observed that where tissues and blood stood for 24 hours, even on ice, higher values for ammonia were obtained.

In view of the demonstrated inaccuracy of the analytical method upon which the conclusions of the Petersburg school were based, Horodyński, Salaskin, and Zaleski (15) considered it necessary to repeat experiments designed particularly to settle the essential question of the excess ammonia of the portal blood. They certified the absolute accuracy of the modified Nencki-Zaleski method, and reported the following average for ammonia (mg. of ammonia in 100 gm. of blood): normal dogs, arterial

blood, 0.41; fasting dogs, arterial blood, 0.42; normal dogs, portal blood, 1.85; fasting dogs, portal blood, 1.29.

In 1902, Folin (16) revived Boussingault's (5) idea of quantitatively removing ammonia from solutions by aeration and adapted it to the estimation of ammonia in urine and other animal fluids. An important detail of Folin's new method was the use of sodium carbonate and sodium chloride to liberate the ammonia, since he found that blood is rich in labile organic compounds from which even weak alkalies, as calcium or magnesium hydroxide, can split off ammonia by hydrolysis at room temperature. Folin employed 50 cc. of blood which were kept cold by ice during the 5 hour aeration, and estimated the ammonia by titration of the excess acid into which it was aerated. He found in the arterial blood of dogs 0.5 to 0.6 mg. of ammonia per 100 gm.

The aeration method did not at once (nor has it to this day) displaced the vacuum distillation technique. Beccari (17), employing the latter procedure, and estimating the ammonia in the distillate as the chloroplatinate, obtained on three dogs the average value of 0.8 mg. of ammonia in 100 gm. of blood.

Voegtlin and King (18), without giving experimental data, say: "The large output of ammonium salts in the urine, as well as high ammonia content of the blood, in clinical conditions involving acidosis, suggested that these salts may play an important rôle in producing the symptoms of these diseases."

Wolf and Marriott (19), using protein-free blood filtrates and a vacuum distillation-titration procedure, found from 1.4 to 4.9 mg. of ammonia nitrogen in 100 cc. of fresh ox blood. Employing Folin's aeration-titration technique, Carlson and Jacobson (20) obtained on normal cats an average of 1.57 mg. of ammonia per 100 cc. of blood, and on normal foxes, 2.388 mg.; on thyroparathyroidectomized cats and foxes, 2.533 and 3.561 mg., respectively. With this same method, Greenwald (21) was unable to obtain satisfactory duplicates, but concluded from his experiments that there is no increase in the blood ammonia of dogs during tetany following parathyroidectomy. Carlson and Jacobson (22), estimating the ammonia by Nessler's reagent instead of titration, following aeration, obtained much smaller values for the blood ammonia than they had previously found and were unable to find any marked or constant increase after parathyroidectomy. Hopkins and Denis (23), by aerating and titrating, found in normal blood from 0.6 to 3.2 mg. of ammonia per 100 cc.

Medwedev (24) made use of a modified vacuum distillation-titration process in which sodium carbonate was employed to liberate the ammonia from the blood. He found that blood, oxalated and preserved under aseptic conditions for 24 hours, shows a significant increase in its ammonia content. When the analysis was undertaken within 10 to 12 minutes after collection he found as the average value in mg. per 100 gm. of blood: in normal dogs, 0.56; in thyroparathyroidectomized dogs, 0.79; and in long fasted dogs, 1.81. The most interesting part of Medwedev's work is his discussion of the changes in the blood ammonia on standing under aseptic

conditions. Thus, in the blood of normal dogs there is an initial slow increase which becomes accelerated in the manner of an autocatalyzed reaction. In the blood of thyroparathyroidectomized dogs there is a much more rapid increase beginning immediately after collection; but in either case, at the end of 24 hours the ammonia has become constant and remains stationary at about the same level. In the blood of long fasted dogs, on the other hand, there is a slow, progressive decrease during the first 6 to 8 hours, after which a slow, progressive increase sets in and continues for 20 to 25 hours but never quite reaches the original value. Medwedew concluded that in blood there is an equilibrium between ammonia and a certain quantity of substance or substances which decompose with the liberation of ammonia. These reactions are catalyzed by a deamidase and a synthetic enzyme of the plasma, while the cells also contain a deamidase. In normal blood the two antagonistic enzymes of the plasma are in equilibrium, but after collection there is a diffusion of the cell deamidase. In the blood of thyroparathyroidectomized dogs the synthetic enzyme of the plasma is diminished in concentration or repressed. While in the blood of fasted dogs the synthetic enzyme at first predominates, but is finally balanced by the diffusion of the cell deamidase. Medwedew believed that these various relations are so constant that they may be formulated mathematically.

In 1912, Folin and Denis (1) published a notable contribution to the subject of ammonia in blood, and reported values far lower than any previously found. The essential details of their analytical method were: the use of small amounts, 5 to 10 cc. of blood; rapid and short aeration, 20 to 30 minutes; estimation of the ammonia by use of Nessler's reagent instead of by titration. In the carotid blood of cats they found from 0.03 to 0.08 mg. of ammonia nitrogen per 100 cc. Further they found that the ammonia of the mesenteric vein of the small intestine is not materially greater, and may be less, than in the portal vein; whereas the ammonia of the mesenteric vein of the large intestine is invariably greater than that in the portal vein. Folin and Denis therefore came to the conclusion that the large intestine (because of the bacterial action therein) is the chief or most constant source of the ammonia found in portal blood, and state: ". . . the portal ammonia is hereby largely robbed of the peculiar interest which has attached to it for the past fifteen years, and since the amount of ammonia in other blood is almost infinitesimal under ordinary normal conditions this too becomes a rather unimportant feature of normal metabolism." They point out that: "The blood decomposes spontaneously (and particularly in the presence of alkalies capable of setting free the ammonia) at all temperatures even when kept on ice. The ammonia thus produced by decomposition in the course of a few hours is much greater than the preformed ammonia present in the strictly fresh blood. . . ."

The values found by Folin and Denis have not gone unchallenged. Matthews and Miller (25) found with the same technique 0.35 mg. of ammonia per 100 gm. of arterial dog blood. Denis (26) reported from 1.0 to 5.5 mg.



of ammonia nitrogen in 100 gm. of blood from various species of fish, but states that further (unpublished) observations on the ammonia content of normal and pathological human blood have shown that ammonia is present to the extent of only a fraction of a mg. per 100 gm. of blood. Jacobson (27) used larger quantities of blood and aerated for 4 hours, estimating the ammonia by Nesslerization, and obtained values ten to twenty times as large as those of Folin and Denis. Rohde (28) carried out vividification experiments on dogs and determined the ammonia in the dialysate when equilibrium had been reached between the dialysate and the blood. In one experiment the dialysate contained 0.18 mg. of ammonia nitrogen per 100 cc.; in a second experiment, 0.30 mg. Rohde also made direct analyses of dog's blood drawn with aseptic precautions, employing Folin's original aeration-titration procedure, aerating for 3 hours, with the addition of saturated sodium carbonate solution to liberate the ammonia. When aeration was begun immediately and carried out at room temperature the value obtained was 0.72 mg. per 100 cc. of blood; when the aeration cylinder was placed in ice the value fell to 0.28 mg. The same blood after having stood for 24 hours on ice, with chloroform and toluene (at the end of which time bacteriological examination gave negative cultures), gave 1.78 mg. of ammonia nitrogen per 100 cc. when aerated at room temperature, and 0.44 mg. when the aeration cylinder stood in ice water during the analysis. Since Rohde found no increase in the ammonia content of the dialysate even on prolonged standing, she concluded that blood contains labile substances which easily split off ammonia (thus confirming Folin, and Medwedew), and that these are to be sought in the non-dialyzable constituents of the blood. Gettler and Baker (29) found, by the Folin-Denis method, from 0.4 to 1.1 mg. of ammonia nitrogen in 100 cc. of normal human blood, with an average for 30 cases of 0.51 mg. Bang (30) aerated protein-free blood filtrates and determined the ammonia by titration. In the blood of normal rabbits he found 0.81 to 1.27 mg. of ammonia nitrogen per 100 gm. He says of these values: "...sind sie bedeutend höher als Folin's Normalwerte, die jedoch unmöglich richtig sein können." Bang found in the blood of a rabbit which showed symptoms of acute poisoning, following the ingestion of 10 gm. of urea 2 hours before, 6.25 mg. of ammonia nitrogen per 100 gm. In other rabbits which had received lethal doses of ammonium carbonate, Bang found from 3.8 to 8 mg. of ammonia nitrogen per 100 gm. of blood. Henriques and Christiansen (31) pointed out the doubtful validity of all experimental data and deductions therefrom, including those of Medwedew, which depend upon vacuum distillation-titration analyses. Furthermore, "Folin und Denis' sehr niedrige Werte (0,03 mg. oder 'Spuren') müssen als irrtümlich betrachtet werden." The analytical procedure employed by Henriques and Christiansen was as follows: 20 cc. of the freshly drawn blood are diluted at once with 4 volumes of ethyl alcohol (to prevent foaming and bacterial action), sodium carbonate is added, and the mixture aerated for 3 hours into dilute sulfuric acid. The content of the receiving flask is then made up to 80 cc. with distilled water and the alcohol distilled off. Sodium hydroxide is

next added and the ammonia distilled through a silver tube condenser into 5 cc. of 0.005  $\times$  sulfuric acid. The excess acid is titrated indirectly by  $\times/280$  sodium thiosulfate (1 cc. = 0.05 mg. of N) using potassium iodate and starch indicator. In arterial blood they found from 0.15 to 0.38 mg. of ammonia nitrogen per 100 cc., and in portal blood from 0.25 to 0.91 mg. They observed little variation in the ammonia content of blood from different animals, and no difference between venous and arterial blood. The ammonia content of blood which stood for a short time was observed to increase, and aeration at temperatures slightly above 16° C. gave higher values than normal. The introduction of large quantities of urea or ammonium salts into the alimentary canal of rabbits did not give uniform results. In those rabbits which showed no symptoms of poisoning there was only a slight rise in the blood ammonia, whereas convulsions were always attended by a marked rise, in one instance reaching 7.38 mg. of ammonia nitrogen per 100 cc. of blood. Gad-Andersen (32) found that the concentrations of ammonia in muscle and blood are identical. His estimation of ammonia was made by a new micro method which was not described. Values for various muscles of from 0.3 to 0.8 mg. of ammonia nitrogen per 100 gm. of tissue were found. All earlier values for muscle, which were usually about twenty-five to thirty times higher than for blood, were due to a transformation of urea into ammonia after death. This transformation may be prevented by the method described. When muscle tissue was allowed to stand under conditions favorable to the transformation, the sum of the urea nitrogen and ammonia nitrogen remained constant, although the initial values of the two were reversed. Morgulis and Jahr (33) absorbed the ammonia from protein-free filtrates by permutit, from which the ammonia was subsequently liberated by sodium hydroxide, and Nesslerized. By adding known amounts of ammonia to the blood and to a control they were able to make the color comparison in an ordinary colorimeter. They found from 0.18 to 0.30 mg. of ammonia per 100 gm. of blood. Myers (34) aerated the ammonia from blood into a definite volume of 0.2  $\times$  sulfuric acid containing 0.05 mg. of ammonium sulfate per cc. This permitted Nesslerization and estimation in an ordinary colorimeter. Myers reported in the blood of various invertebrates from 0.00 to 1.05 mg. of ammonia nitrogen per 100 cc., with a general rough parallelism between the ammonia nitrogen and total non-protein nitrogen. In whale blood (which was obtained for analysis 3 to 4 hours or longer after death) Myers found 2.4 to 14.5 mg. of ammonia nitrogen per 100 cc. Myers corroborates Barnett's claim that there is a decided increase in the ammonia of blood on standing.

The only reported values of ammonia in blood of the same order as those found by Folin and Denis were obtained by Barnett (35) who devised an aeration-micro-titration procedure. Using capillary pipettes, methyl red indicator, and a sodium acetate-acetic acid mixture for a fixed comparative end-point, Barnett was able to obtain a sharp end-point with 0.005 cc. of 0.005  $\times$  alkali. He found in oxalated human blood, whose analysis was begun within 1 to 3 minutes after drawing, from 0.00 to 0.05 mg. of ammonia

per 100 cc. The same bloods 30 minutes later showed from 0.07 to 0.15 mg. of ammonia per 100 cc. Barnett stated that the results of analyses of ammonia in blood "are largely merely a measure of the extent to which the labile ammonia-yielding bodies of the blood have been disintegrated. . . . no means of overcoming this difficulty has been devised, and the best we can do is to minimize the error from the ammonia increase by beginning the aeration as soon as possible after the blood is drawn, and completing the analysis as rapidly as possible." Barnett and Addis (36), employing the above micro-titration technique, found in the blood of rabbits, following large doses of urea given by mouth, or directly into the bowel, or intravenously, marked increases in the ammonia content. In one instance the blood ammonia rose to 11 mg. in 100 cc.

If the extremely low values of blood ammonia reported by Folin and Denis, and Barnett, are accepted it becomes a very interesting problem to account for the relatively enormous quantities of ammonia excreted even in normal urine. A calculation will make clear the nature of the problem: If the rate of blood flow through the kidneys is 150 cc. per minute per 100 gm. of kidney tissue (37), and the total weight of kidney tissue is 300 gm., then the 24 hour volume of blood passing through the kidneys is 648 liters. Assuming that 100 cc. of arterial blood contains 0.1 mg. of ammonia nitrogen, and that this is *completely* removed, the total output of ammonia nitrogen in the urine for 24 hours would be 0.648 gm. This value is very close to that actually found for the normal, average individual, but the assumed figure for arterial blood is appreciably higher than that which we usually found, and it is scarcely conceivable that the kidney is 100 per cent efficient in the excretion of ammonia. Further, so low a concentration of blood ammonia is entirely inadequate to account for the high urinary ammonia found in various pathological conditions or after acid ingestion. It would seem, therefore, that one of the following possibilities must be true: *First*, the blood ammonia is higher than the value assumed above; *second*, ammonia is not present as such in the blood, but is in a loose combination readily split by the kidney although not easily dissociated under the conditions of analyses hitherto employed; or *third*, the kidney itself forms the ammonia which it eliminates. An investigation of these several hypotheses was the object of the work here reported.

## ANALYTICAL PROCEDURE.

Of the various procedures which have been proposed for the estimation of ammonia in blood the combination of aeration and Nesslerization seems least open to criticism. Salkowski (38) very early pointed out that milk of lime effects a decomposition of protein which invalidates ammonia determinations in protein-containing mixtures. Folin and Denis (1) state that "when distillation methods are applied, whether in the vacuum or otherwise, the determination becomes little less than a measure of the decomposition." Matthews and Miller (25) have well expressed the difficulties inherent in the estimation of small amounts of ammonia by titration: "It is well known that most indicators do not react with the greatest precision and therefore are not applicable when very small amounts are to be taken into account. This is especially true with ammonia, which is likely to form hydrolyzed salts with the indicator and thus give an indefinite end-point. In fact when not more than 0.5 mgm. of ammonia is absorbed in 100 cc. of  $\frac{N}{10}$   $H_2SO_4$  solution, it is impossible to titrate with sufficient accuracy to even approximate the actual amount, the limits of error being several times greater than the amount of ammonia to be determined . . . . Nessler's method . . . . is known to be accurate enough to distinguish 0.01 mgm. of ammonia and is therefore at least ten times more accurate than the titration methods heretofore ordinarily used. In fact it is the only known method accurate enough to determine quantities of ammonia ranging from 0.01 to 0.05 mgm." Another serious objection to the titration method is the difficulty of keeping standard solutions as dilute as those employed. This applies particularly in such micro-titration technique as that employed by Barnett (35), whose highest value for ammonia in human blood, 0.05 mg. of ammonia in 100 cc., corresponds to about 0.03 cc. of the 0.01 N hydrochloric acid used for the amount (10 cc.) of blood taken for analysis. The direct Nesslerization of protein-free blood filtrates, proposed by Morgulis and Jahr (33), whether or not permutit is used to absorb the ammonia, is open to Folin's (39) criticisms, and is not worthy of detailed consideration.

In connection with the determination of ammonia in blood by the aeration method it is well to bear in mind that this determination involves the measuring of smaller quantities of material than are dealt with in perhaps any other analytical process. Furthermore, the substance to be determined is present in the air of laboratories in appreciable quantity, and is contained in most reagents in detectable amounts. These considerations show that the determination of ammonia in blood can be of sufficient accuracy only when the most rigid precautions are observed at every stage of the process. Indeed it required several weeks of unremitting effort before we were able to obtain distilled water blanks closely approximating the same ammonia-free water without aeration. Rubber stoppers may absorb ammonia in appreciable amounts from the laboratory air, which comes off slowly during the aeration. Triple acid washing of the air prior to its introduction into the blood is necessary for complete removal of the ammonia originally present.

The analytical procedure employed by us is essentially the Folin-Denis method with added precautions of technique which we believe are essential for correct results. The exact procedure is as follows: 5 cc. of oxalated<sup>1</sup> and well mixed blood is aerated for 10 minutes, the ammonia being received in 5 cc. of ammonia-free water<sup>1</sup> containing 2 to 3 drops of 0.1 N hydrochloric acid.<sup>1</sup>

<sup>1</sup> Reagents. Potassium oxalate used to prevent clotting was obtained ammonia-free by repeated recrystallization from ammonia-free water. Ammonia-free water was prepared by distilling dilute sulfuric acid, and collecting only a middle fraction. 0.1 N hydrochloric acid was prepared by diluting concentrated acid from a freshly opened bottle; 3 drops of the dilute acid in 5 cc. of ammonia-free water gave no trace of color with Nessler's reagent. The carbonate-oxalate solution employed was made by dissolving 8 gm. of anhydrous potassium carbonate and 12 gm. of crystalline potassium oxalate in distilled water, boiling down rapidly to half volume, diluting with ammonia-free water, and again boiling down to a small volume, and finally diluting to 80 cc. with ammonia-free water. The caprylic alcohol used was redistilled from a lot obtained from Eimer and Amend; we could detect no effect of the addition of 1 drop of the alcohol in blank determinations. The Nessler reagent was prepared according to the directions of Bock and Benedict. Standards containing 0.01, 0.02, 0.03, 0.04, 0.05, 0.075, 0.10, 0.15, 0.20 mg. of ammonia nitrogen per 100 cc. were readily prepared by dilution of a standard solution of ammonium chloride.

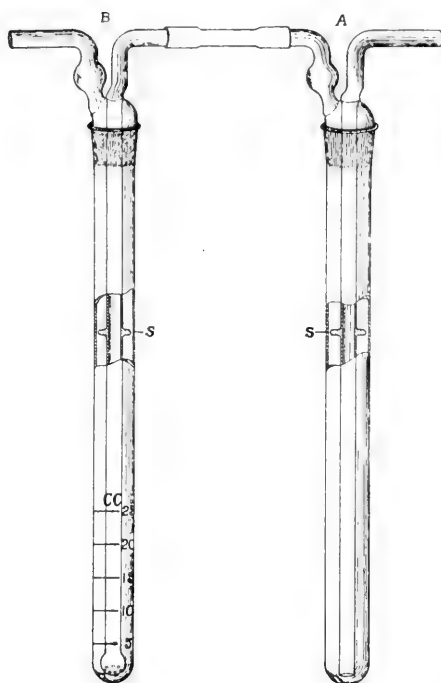


FIG. 1. The tubes are 300 mm. tall, exclusive of stoppers, and are made of good quality *white* glass. The internal diameter of Tube A is 20 mm., of Tube B 19 mm. The ground in stoppers carry the inlet and outlet tubes. (We have found it practically impossible to get absolute blanks when rubber-stoppered tubes are used.) Baffle plates, *S*, of glass are fused to the inlet tubes. These plates have a clearance of about 1 mm., and are very effective in preventing spray being carried over by the air current. They serve also to break the otherwise troublesome "foam rings" which slide up the tubes toward the end of the aeration when most of the caprylic alcohol has been driven out. The baffle plates, together with the height of the tubes, make possible a very rapid aeration. If desired, cotton or glass-wool may be placed in the bulbs of the outlet tubes as a further precaution against the carrying over of spray, but we have not found this necessary or desirable. The tubes are connected by rubber tubing, and any number of sets may be connected in series. This apparatus was made for us by Wm. T. Wiegand, 141 Lexington Ave., New York City.

To liberate the ammonia from the blood we use 1 cc. of a potassium carbonate-oxalate solution.<sup>1</sup> 1 drop<sup>2</sup> of caprylic alcohol<sup>1</sup> in the blood tube suffices during the short aeration to prevent frothing in both the blood tube and the acid collection tube. The special construction of the tubes (Fig. 1) allows an aeration rate of 4 liters per minute, which we obtain with a suction pump. The air is washed through a train of three<sup>3</sup> sulfuric acid absorption bottles to remove ammonia and then through a 1 per cent hydrochloric acid solution to saturate the air with moisture, before reaching the blood tube. When the aeration is completed the solution in the receiving tube is Nesslerized without transferring it,<sup>4</sup> 2 drops of undiluted Nessler reagent<sup>1</sup> being added. The ammonia is then estimated by transverse comparison with similarly and simultaneously Nesslerized standards<sup>1</sup> contained in tubes of the same internal diameter as that of the receiving aeration tube.<sup>5</sup>

The possibility of accurately recovering very small amounts of ammonia added to water by the procedure described was established by the results given in Table I.

Our first experiments with blood were designed to study the effect of standing on the ammonia content of the blood. No aseptic precautions were observed in drawing the blood, which was oxalated and kept in stoppered tubes on ice (except where otherwise noted) until used. Portions of the blood were successively aerated three times in a period of an hour. These samples, of course, stood in contact with the carbonate. Other portions

<sup>2</sup> More than 1 drop of caprylic alcohol may cause turbidity in the Nesslerized solution, particularly if the concentration of ammonia nitrogen is less than 0.05 mg. per 100 cc.

<sup>3</sup> Bock and Benedict (40) have shown that a single acid wash bottle does not completely remove the ammonia from air. This fact does not necessarily discount the use of a single absorption tube for the ammonia of blood, since the amount of ammonia escaping absorption appears to be a factor of the concentration of ammonia in the air.

<sup>4</sup> It is obvious that the inner absorption tube need not be washed down unless the "unknown" is to be diluted beyond its original volume.

<sup>5</sup> Differences of 0.01 mg. of ammonia nitrogen in 100 cc. are readily estimated in this manner, up to 0.20 mg. Higher concentrations than this are compared in an ordinary colorimeter. The standard tubes are graduated to permit dilution of the prepared standards when necessary to obtain intermediate values.

of the same blood were kept on ice and aerated at various times after drawing, as indicated in Table II.

An inspection of Table II will show that there is a slight increase in the ammonia content after standing 30 minutes, but that further standing up to 3 or 4 hours causes no increase in the ammonia. Our results here are in striking disagreement with the conclusions of Folin and Denis (1), Barnett (35), and others, concerning the presence of "labile ammonia-yielding bodies" in blood.

It will be noted however, from Table II, that the first period of standing always yields a small increment of ammonia over that obtained when the blood is freshly aerated. Investigating this

TABLE I.  
*Recovery of Known Amounts of Ammonia Added to Water.*

NH <sub>3</sub> -N per 100 cc.		NH <sub>3</sub> -N per 100 cc.	
Taken.	Found.	Taken.	Found.
mg.	mg.	mg.	mg.
0.02	0.03	0.06	0.06
0.02	0.03	0.07	0.08
0.03	0.04	0.10	0.10
0.03	0.04	0.20	0.20
0.04	0.035	0.40	0.40
0.04	0.07	0.40	0.40
0.05	0.05		

question further, we carried out simultaneous analyses of dog blood and plasma under identical conditions.

From the results given in Table III it will be seen that with plasma there is no appreciable yield of ammonia after the first aeration, while just the opposite result is obtained with corpuscles.

Henriques and Christiansen (31) found that, on a volume basis, blood corpuscles contain from two to four times as much ammonia as the plasma. We believe from our results, however, that the concentration of ammonia is essentially the same in corpuscles and plasma; and we attribute the small increase which we find in blood on standing to hemolysis and perhaps to diffusion of ammonium salts from the corpuscles into the plasma.

To test whether ammonia would be split off from a typical protein under the conditions of our aeration we have carried out



the following experiments. Fresh hen's egg albumin was diluted with ammonia-free water, and 5 cc. portions were aerated immediately after addition of 1 cc. of carbonate-oxalate solution,

TABLE II.  
*The Effect of Standing on the Blood Ammonia.\**

Subject.	Source of blood.	Repeated aeration of a single portion of blood after addition of carbonate-oxalate, and standing at room temperature.				Blood after standing on ice previous to addition of carbonate-oxalate.	
		Interval between drawing blood and first aeration.	NH <sub>3</sub> -N			Time of standing.	NH <sub>3</sub> -N
			First aeration.	Second aeration 30 min. after first.	Third aeration 30 min. after second.		
		min.	mg.	mg.	mg.	min.	mg.
Cat.	Renal vein.	15	0.18	0.05	0.04		
"	Vena cava.	5	0.12	0.06	0.07		
Dog.	Carotid.†	60	0.14	0.02	0.02	150	0.16
"	"	1	0.06			120	0.08
"	Renal vein.	15	0.09	0.02	0.00	120	0.14
"	Vena cava.	1	0.03	0.01	0.00		
"	" "	1	0.16	0.04	0.00	120	0.18
"	Carotid.	1	0.10			150	0.11
"	"	150	0.11	0.03	0.01		
"	Renal vein.	30	0.19			180	0.18
"	" "	10	0.18	0.04	0.01	240	0.28
"	Vena cava.	2	0.07	0.01	0.00	240	0.10
"	Renal vein.	180	0.18	0.03	0.00		
"	Femoral artery.	15	0.04	0.01	0.00	210	0.07
"	Carotid.	1	0.07			120	0.07
"	Renal vein.	15	0.21	0.03	0.01		
"	Vena cava.	5	0.08	0.01	0.00		
"	Carotid.	1	0.08			120	0.10
"	"	120	0.10	0.02	0.01		
"	Renal vein.	10	0.28	0.03	0.01		
"	Vena cava.	5	0.06	0.03	0.01	90	0.08

\* Results are given for 100 cc. of blood.

† This blood stood at room temperature throughout.

again after 30 minutes, and again after a further 30 minute interval. The results are given in Table IV.

The figures reported in Table IV show that egg albumin does not decompose to yield ammonia under the conditions which

we used for blood. These results lend some support to the view that the quantities of ammonia found in blood are preformed, and are not decomposition products formed during the analysis. This latter view remains, however, a possibility.

TABLE III.  
*Ammonia in Whole Blood and Plasma: Effect of Standing.*

Dog.	Source of blood.	NH <sub>3</sub> -N in 100 cc.					
		Blood.			Plasma.		
		First aeration.	Second aeration 30 min. after first.	Third aeration 30 min. after second.	First aeration.	Second aeration 30 min. after first.	Third aeration 30 min. after second.
		mg.	mg.	mg.	mg.	mg.	mg.
1	Vena cava.	0.18	0.05	0.00	0.20	0.00	0.00
2	Carotid.	0.11	0.03	0.01	0.13	0.00	0.00
3	Femoral artery.	0.07	0.03	0.00	0.08	0.01	0.00
4	Mixed systemic.	0.12	0.03	0.01	0.10	0.02	0.00
5	Carotid.	0.10	0.02	0.01	0.12	0.00	0.00

TABLE IV.  
*Experiment 2. Ammonia in Egg Albumin Mixtures.*

No.	NH <sub>3</sub> -N in 100 cc.			Remarks.
	First aeration.	Second aeration 30 min. after first.	Third aeration 30 min. after second.	
	mg.	mg.	mg.	
1	0.05	0.00		26 cc. of fresh egg albumin diluted to 44 cc.
2	0.00		0.01	Fresh egg albumin diluted four times.
3	0.07	0.00	0.00	" " " " to double volume.
4	0.03	0.00	0.00	" " "

The next point studied was concerned with the question as to whether minute amounts of ammonia added to shed blood can be recovered quantitatively. This question is directly related to the broader question as to whether ammonia may be transported in the blood in a complex combination from which the ammonia cannot be liberated by simple treatment with carbonate-

oxalate mixture. While this latter question is obviously difficult to study directly, it seems probable that if the blood possesses the power of combining ammonia, then minute amounts of ammonia added to blood should not be completely recovered.

In connection with any theory of a complex ammonia combination in blood it should be borne in mind that any combination of ammonia from which the base could not be liberated by treatment with sodium carbonate would presumably defeat the object of ammonia formation in the organism, if we assume that the ammonia is produced for the purpose of acid neutralization.

In testing the recovery of added ammonia we made use of ox blood. Simultaneous analyses were carried out with and without the addition of small amounts of ammonia nitrogen.

The figures given in Table V show that ammonia added to blood can be completely recovered within the limits of accuracy of the method.

The next experiment was planned to find out whether the blood can yield ammonia to neutralize added acid. Ammonia in such a combination would be readily available for the needs of the organism. The following typical experiment shows that blood does not yield ammonia to neutralize added acid.

Fresh ox blood gave on analysis 0.08 mg. of ammonia nitrogen per 100 cc. To 10 cc. of this blood was added 1 cc. of ammonia-free isotonic saline solution, and to a second 10 cc. portion of blood was added 1 cc. of a 10 per cent lactic acid solution. The two mixtures were then incubated in a water bath at 38–40°C. At the end of 30 minutes the blood-saline mixture gave 0.08 mg. of  $\text{NH}_3\text{-N}$  per 100 cc., and the blood-acid mixture gave 0.09 mg. of  $\text{NH}_3\text{-N}$  per 100 cc. 30 minutes later the values were 0.09 and 0.08, respectively.

A study of the blood ammonia concentration in animals where the urinary ammonia output is higher than normal was made upon phlorhizinized dogs which were available from other experiments. These dogs had received daily injections of 1 gm. of phlorhizin in oil over long periods, and blood was taken for analysis when the animals were in the late stages of the poisoning.

The results given in Table VI show that even where the ammonia content of the urine is markedly increased, there is no increase in the ammonia content of the blood. (Compare figures on normal dogs, Table II.)

TABLE V.

*Recovery of Ammonia Added to Blood. Results Given in Terms of  $\text{NH}_3\text{-N}$  per 100 cc. of Blood.*

No.	Original.*	Added.	Found.	Difference.	Remarks.
	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>	
1	0.08	0.15	0.23	0.00	Added $\text{NH}_4\text{OH}$ . Aerated immediately.
1	0.075	0.075	0.18	0.03	“ “ “
1	0.075	0.15	0.23	0.005	Added $\text{NH}_4\text{Cl}$ . Aerated immediately.
2	0.20	0.20	0.37	0.03	Added $\text{NH}_4\text{OH}$ in isotonic saline solution. Aerated at once.
2	0.20	0.20	0.39	0.01	Added $\text{NH}_4\text{Cl}$ in isotonic saline solution. Aerated at once.
2	0.20	0.20	0.33	0.07	Added $\text{NH}_4\text{OH}$ in isotonic saline solution. Stood 1 hour at room temperature before aeration.
2	0.20	0.20	0.37	0.03	Added $\text{NH}_4\text{Cl}$ in isotonic saline solution. Stood 1 hour at room temperature before aeration.
2	0.26	0.20	0.37	0.09	Added $\text{NH}_4\text{OH}$ in isotonic saline solution. Stood 2 hours at room temperature before aeration.
2	0.33	0.20	0.45	0.08	Added $\text{NH}_4\text{OH}$ in isotonic saline solution. Stood 24 hours in ice box before aeration.
3	0.20	0.20	0.38	0.02	Added $\text{NH}_4\text{OH}$ in isotonic saline solution. Aerated at once.
3	0.20	0.20	0.42	0.02	Added $\text{NH}_4\text{OH}$ in isotonic saline solution. Stood 1 hour at room temperature before aeration.
4	0.36	0.25	0.66	0.05	Added $\text{NH}_4\text{OH}$ . Stood 30 minutes at room temperature before aeration.
4	0.36	1.00	1.41	0.05	Added $\text{NH}_4\text{Cl}$ . Stood 30 minutes at room temperature before aeration.
5	0.09	0.094	0.20	0.016	Added $\text{NH}_4\text{-lactate}$ in isotonic saline solution. Aerated at once.
5	0.09	0.188	0.26	0.018	“ “ “

TABLE V--*Continued.*

No.	Original.*	Added.	Found.	Difference.	Remarks.
	mg.	mg.	mg.	mg.	
5	0.10	0.094	0.22	0.026	Added NH <sub>4</sub> -lactate in isotonic saline solution. Stood 30 minutes at 38-40° C. before aeration.
5	0.11	0.094	0.22	0.016	Added NH <sub>4</sub> -lactate in isotonic saline solution. Stood 1 hour at 38-40° C. before aeration.

\* The values in this column do not represent the average preformed ammonia nitrogen in fresh ox blood. The blood used was brought to the laboratory from a slaughter house, and the higher values found undoubtedly are to be attributed to bacterial action or to the presence of foreign material. We have found as the average value for 15 slaughter house bloods, when the analyses were made as soon as received, 0.13 mg. of ammonia nitrogen per 100 cc., but the conditions of handling and drawing this blood were not rigidly controlled.

TABLE VI.

*Experiment 6. Ammonia in the Blood of Phlorhizinized Dogs.*

Dog.	Source of Blood.	NH <sub>3</sub> -N in 100 cc. of blood.	NH <sub>3</sub> -N of total N in previous 24 hour urine.	Plasma CO <sub>2</sub> c. p.
		mg.	per cent	vol. per cent
6	Femoral artery.	0.075	11.0	
9	Carotid.	0.07	6.3	38.6
23	Femoral artery.	0.07	6.5	47.6
26	Jugular.	0.09	4.0	38.1
27	"	0.05	4.6	54.1

It seemed desirable next to test the question as to whether there is accumulation of ammonia in the blood following double nephrectomy, or after ligation of both ureters. Similar experiments have been reported for dogs by Winterberg (41) and for goats by Henriques and Christiansen (31). These investigators failed to find increased ammonia in the blood under the conditions cited. Since with the kidneys extirpated (or after ligation of both ureters) acid formation must be going forward, while ammonia cannot leave the organism, we deemed this question of sufficient import-

ance to warrant full repetition of the experiments on dogs. Our experiments in this connection were carried out as follows.

The dogs of this series were operated under ether anesthesia. Through a midline incision in the abdominal wall the renal vessels were ligated and the kidneys removed; in other cases only the ureters were tied off. We observed that those dogs, in which only the ureters were ligated and the kidneys left, survived the operation somewhat longer than those dogs whose kidneys were extirpated.<sup>6</sup> No convulsions or other typical "uremic" symptoms were observed in the operated animals other than slight tremors in the extremities, rapid and shallow breathing, and rapid and labored heart action. Blood was taken for analysis in most animals when it appeared that the animal was near death. In the oxalated blood the corpuscles settled with extraordinary rapidity, leaving a plasma of light yellow color.

An inspection of the results given in Table VII shows (in agreement with the previous investigators above cited) that in spite of total absence of kidney function in dogs there is no accumulation of ammonia in the blood. In fact some of these experimental animals gave us the lowest figures for ammonia in the blood which we have found. This failure to find any accumulation of ammonia in the blood after extirpation of the kidneys is, we believe, a unique finding for this substance as compared with any other constituent of both blood and urine, and we believe that this finding necessitates the conclusion drawn later in this paper concerning the origin of ammonia in the organism.

In view of the facts shown above, especially the findings that there is no accumulation of ammonia in the blood in phlorhizinized dogs, or in dogs without functioning kidneys, we were led to the conclusion that the kidneys themselves must produce the urinary ammonia. That the kidneys may perform an active synthetic function is not a new idea (Bunge and Schmiedeberg, 43), but so far as we know the production of ammonia has not hitherto been ascribed to the kidney.

It seemed probable that if ammonia production takes place in the kidney, this organ would not excrete every trace of the ammonia formed, and we might then expect to find the blood of

<sup>6</sup> In this we do not agree with Jackson (42).

the renal vein richer in ammonia than the systemic blood. We have therefore carried out experiments in which we compared the ammonia content of the carotid blood with that of the renal vein. In most cases we also included determinations of the ammonia content of blood from the vena cava taken posterior to where the renal veins enter this vessel.

In collecting the several bloods for analysis in this experiment we have proceeded as follows: The animal was anesthetized with ether, and a cannula placed in the carotid artery. In some cases the arterial blood was drawn first, in other cases last; we have not

TABLE VII.

*Experiment 7. The Blood Ammonia in Nephrectomized Dogs, and in Dogs with Ligated Ureters.*

Dog.	Source of blood.	Time after operation.	Per 100 cc. of blood.		Plasma CO <sub>2</sub> c. p.	Remarks.
			NH <sub>3</sub> -N	N-P-N		
		hrs.	mg.	mg.	vol. per cent	
7	Femoral artery.	44	0.06	200		Kidneys extirpated.
8	" "	46	0.08	182		Ureters tied.
8	" "	71	0.10	197		" "
14	" "	42	0.03	172	38.2	Kidneys extirpated.
18	Carotid.	47	0.03	162	57.1	Ureters tied. Autopsy disclosed severe hemorrhage due to bursting of capsule of one kidney.
21	"	47	0.12	186	36.2	Ureters tied.

found that the order of taking the blood is material. The abdomen was opened by a midline incision, and the vena cava and the renal vein on one side exposed. Blood was then taken from the renal vein (we found the most convenient method of taking this blood was by the use of a curved needle attached by a piece of rubber tubing to a 25 cc. pipette containing potassium oxalate, the blood being drawn into the pipette by carefully regulated suction). The point of the needle was introduced into the vein toward the kidney. The renal vein was tied off (to prevent hemorrhage), and a ligature placed around the vena cava just behind the renal veins. Blood was now quickly drawn from the vena cava, caudad

to the ligature, using a similar technique to that described for the renal vein.

It will be noted from the experiments reported in Table VIII that the blood of the renal vein is invariably much higher in ammonia content than the systemic arterial or venous blood.

TABLE VIII.

*Experiment 8. Comparison of Ammonia Content of Systemic and Renal Venous Blood.*

Subject.	Sex.	NH <sub>3</sub> -N in 100 cc. of blood.			Remarks.
		Carotid.	Vena Cava.	Renal vein.	
		mg.	mg.	mg.	
Cat 1	Male.	0.08		0.20	Ether anesthesia.
" 2	Female.	0.08		0.26	Chloretone anesthesia. Cat pregnant.
" 3	Male.	0.12	0.12	0.22	Ether anesthesia.
" 4	Female.	0.11	0.10	0.27	" " Cat pregnant.
" 5	Male.	0.12	0.12	0.18	Ether anesthesia.
Average.....		0.102	0.113	0.226	
Dog 10	Female.	0.08		0.22 (L) 0.18 (R)	Ether anesthesia.
" 11	Male.	0.06	0.03	0.09	" "
" 12	"	0.075	0.16	0.12	" "
" 13	"	0.10	0.07	0.19 (R) 0.18 (L)	" "
" 15	"	0.07	0.08	0.21	" "
" 24	Female.	0.14		0.25	" " Dog in last stages of phlorhizin poisoning.
" 25	"	0.13		0.18	" " "
" 27	"	0.05*		0.14	" " "
Average.....		0.088	0.085	0.176	

\* Blood of jugular vein.

The blood from the renal vein averages twice as much ammonia as does the blood from other sources.

These differences are so marked as to admit of only one interpretation; *viz.*, that the kidney, instead of excreting ammonia from the blood, forms the ammonia which it excretes, while at



the same time it contributes a small amount of ammonia to the blood. No theory of concentration (by abstracting water) can possibly explain the differences in ammonia content which we have found, since to explain the ammonia increase in the renal vein on such a basis we should have to assume a 50 per cent concentration of the blood in a single passage through the kidney.

As a corollary of the view that the kidney is the center of ammonia production in the body we should expect that acid or alkali introduction into the organism should have no detectable effect on the ammonia content of the systemic blood, while the ammonia content of the blood of the renal vein might be expected to show a slight increase in ammonia after acid introduction and a slight decrease after alkali ingestion. These results would be expected because as the kidney makes more or less ammonia we might expect some corresponding change in the ammonia escaping into the renal blood. There is no reason to believe that the slight changes which might be produced in the ammonia concentration of the blood of the renal vein should be reflected in the blood of the general circulation, for we must assume that the ammonia of the systemic blood represents an equilibrium state between the ammonia which comes into the circulation by way of the renal veins (and possibly traces of ammonia from the intestinal circulation which pass the liver) and the transformation of this ammonia into urea. If ammonia were formed in the organism in appreciable amounts elsewhere than in the kidney, we should expect injection of acid into the circulation to be followed by a definite increase in the ammonia of the general systemic blood. The opposite change might be expected as a result of alkali treatment. The experiments conducted in this connection were as follows.

#### *Effect of Acid Injection.*

The procedure was as follows: The animal was etherized and cannulae were placed in the carotid artery and jugular vein. A sample of carotid blood was taken and the ammonia determined. 1.0 N hydrochloric acid was then run from a burette into the jugular vein at the rate of about 1 cc. per minute, until the animal showed severe symptoms of dyspnea. As rapidly as possible then blood was again taken from the carotid artery, and from

the renal vein and vena cava. The ammonia in each of these bloods was determined, and in the carotid blood, the plasma  $\text{CO}_2$ -combining power was also determined.

*Effect of Alkali Injection.*

In one series of dogs we attempted to eliminate ammonia from the urine by means of sodium bicarbonate in olive oil injected subcutaneously. The urinary ammonia was determined in daily catheter specimens, and while it did not completely disappear it fell on several days to very low values. The ammonia and  $\text{CO}_2$ -combining power of the jugular blood was followed during the course of the injections, the specimens of blood being taken through a needle inserted through the skin into the vein.

TABLE IX.

*Experiment 9. Effect of Acid Injection on Blood Ammonia.*

Dog.	NH <sub>3</sub> -N in 100 cc. of blood.					Remarks.
	Before.	After.				
	Carotid.	Carotid.	Vena cava.	Renal vein.	Carotid plasma CO <sub>2</sub> c. p.	
	mg.	mg.	mg.	mg.	vol. per cent	
16	0.16	0.075	0.06	0.28	13.4	30 cc. of 1.0 N HCl injected.
17	0.075	0.085	0.10	0.25	18.5	45 cc. of 1.0 N HCl injected.

In one dog, blood taken from the jugular vein gave 0.07 mg. of ammonia nitrogen per 100 cc. The dog was then etherized and 2 per cent  $\text{NaHCO}_3$  solution run into the vein through a cannula. After 2 hours, when 196 cc. of the bicarbonate solution had been injected, the urine (by catheter) was ammonia-free. At this time, femoral arterial blood gave 0.07 mg., and renal venous blood 0.18 mg. of ammonia nitrogen per 100 cc.

From Tables IX and X it is plain that acid or alkali injection have no influence on the ammonia content of the systemic blood. There is some evidence of an effect on the ammonia content of the blood of the renal vein, but further experiments would be required to warrant any definite conclusion here.

TABLE X.

*Experiment 9. Effect of Alkali Injection on Blood Ammonia. NaHCO<sub>3</sub> in Olive Oil.*

Date.	Urine.		Blood.				NaHCO <sub>3</sub> injected.
	Reaction to litmus.	NH <sub>3</sub> -N	Jugular.		NH <sub>3</sub> -N in 100 cc. of renal venous blood.	NH <sub>3</sub> -N in 100 cc. of vena cava blood.	
			CO <sub>2</sub> C. P.	NH <sub>3</sub> -N in 100 cc.			
Dog 19.							
May 5	Basic.	per cent 0.038	vol. percent 47.7	mg. 0.10			gm. 2
" 6	"	0.010	60.0	0.05			3
" 11	"	0.115	52.9	0.07			2
" 12	"	0.030	*	0.06	0.13	0.08	
Dog 22.							
May 20	Acid.	0.152					2
" 23	Basic.	0.068	66.8	0.07			2.5
" 24	Acid.	0.050					
" 25	Basic.	0.040					2.5
" 26	"	0.036					4
" 27	"	0.004					4
" 28	"	0.014	80.2	0.07	0.16	0.06	

\* CO<sub>2</sub>-combining power of carotid blood plasma, under ether anesthesia, was 39.3 volumes per cent.

## DISCUSSION.

If we accept the view that ammonia production takes place in the kidney as part of its excretory function, it would seem that certain facts in regard to acidosis may be more readily understood. Under the commonly accepted view that neutralization of acids by ammonia is a function of the organism in general, or of the liver, it would seem very difficult to understand such acidosis (depletion of the alkali reserve) as frequently occurs in nephritis, where there is no marked increase in acid production. Even the increase in acid phosphate in the blood reported in some of these cases affords no explanation of depletion of the alkali reserve, since ammonia should be available for neutralization of any circulating acid.

If, however, we look upon the kidney as the seat of ammonia production, depletion of the alkali reserve becomes readily understandable under certain definite conditions. If ammonia is not available within the organism the acids must be transported wholly in combination with the fixed bases, or with protein. A depletion of the alkali reserve of the blood could therefore arise under any one of three definite conditions.

1. Introduction of acid radicles into the blood stream more rapidly than the normal kidney can eliminate them, or can make ammonia to combine with them while eliminating them.

2. If the kidney becomes defective in its power to eliminate acid radicles, and thus to maintain them at a minimal level in the blood, a depletion of the alkali reserve would result, since the acid radicles would remain in the circulation in abnormal amounts, and would have to be neutralized by the fixed bases or protein. This condition might well result with a kidney still normal in its power of ammonia production. Such ammonia is available for the needs of the organism only as acid radicles are excreted.

3. A depletion of the alkali reserve of the blood would result should the kidney become defective in its power of ammonia formation. Even should such a kidney remain normal in its power of excreting acid radicles, the organism would lose base excessively during the excretion of the acid.

It would appear that the first of these three forms of acidosis occurs, if at all, in diabetes. Very probably either or both of the two latter forms occur in nephritis. It seems from our results that acidosis in the sense of depletion of the alkali reserve is primarily a kidney disease.

Although Wakeman and Dakin (44) came to the conclusion that the formation of urea in the animal body is an irreversible process, we believe that urea is the most probable precursor of ammonia in the kidney. It has been frequently demonstrated that the urinary ammonia is increased at the expense of urea, and unless we assume a conversion of urea into ammonia by the kidney we should have to assume the transportation in the blood of some intermediate product between urea and ammonia, or of some "complex ammonia compound." Our work has rendered either of these views very improbable. It is, of course, also possible that the kidney is active in deamination of amino-acids, and that excreted ammonia is supplied from this source.

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## THE MECHANISM OF REDUCTION OF NITRATES AND NITRITES IN PROCESSES OF ASSIMILATION.

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(Received for publication, July 30, 1921.)

The mechanism of reduction of nitrates of the alkali metals to ammonia and the formation of amino nitrogen in biochemical syntheses from inorganic nitrogen compounds have not been explained satisfactorily. Some investigators explain the transformation by assuming a direct reduction of the nitrates to ammonia by action of nascent hydrogen, while others assume an intermediate reduction of the nitrate to nitrite, from which, as the reduction proceeds, ammonia is produced. The exact chemical procedure by which bacteria or molds are able to produce ammonia or nitrites from alkali nitrates also has not been satisfactorily explained.

Schimper,<sup>1</sup> the botanist, has been able to demonstrate experimentally that the reduction of nitrates in green leaves is connected intimately in some manner, not only with the influence of light, but also with the action of iron compounds in the leaves. This observation was so interesting to the writer that it led him to an investigation of the question whether iron actually takes part in the reduction of nitrates by means of bacteria. This work has proved very productive and, as a matter of fact, experiments have shown conclusively that the *cholera bacillus*, which possesses extraordinary reducing power for nitrates, has the ability to accumulate iron<sup>2</sup> in its organism, and its reducing power may possibly be a function, not only of its ability to absorb oxygen by respiration, but also of its iron content.

The data revealed through this biological research, which was interrupted by the War and political disturbances in Europe,

<sup>1</sup> Schimper, A. F. W., *Bot. Z.*, 1890, xlvii, 73.

<sup>2</sup> Unpublished data.

warranted a purely chemical investigation of the reduction of inorganic nitrates by means of iron salts. The opportunity to continue this work has now been offered to me, and, as a result, the study of this interesting problem has been taken up.<sup>3</sup>

An interesting paper by Menaul which recently appeared in this Journal,<sup>4</sup> brings up for discussion a very important biochemical change. This investigator describes the action of formaldehyde on saltpeter in aqueous solution when exposed to sunlight, and the observation is made by him that in such solutions small quantities of hydrocyanic acid can be detected easily. This quite remarkable action of sunlight on nitrates was observed by the writer several years ago<sup>5</sup> and this preliminary paper is now contributed to bring the results of this work before the American reader, and at the same time to present a summary of the principal results of his earlier investigations on the photochemical reduction of nitrates and the reduction of nitrates and nitrites with iron salts, which have appeared in various scientific publications during the last 10 years.<sup>6</sup> This summary will be presented in three parts as follows: (1) Reduction of nitrates of alkali metals by means of light and also iron salts; (2) Reduction of nitrites of alkali metals by means of light and also iron salts; and (3) Synthesis of organic compounds containing nitrogen from inorganic compounds of nitrogen.

#### *The Reduction of Nitrates.*

In the course of researches on the photochemical decomposition and synthesis of nitrates and nitrites, the observation was made, for the first time, that one oxygen atom in nitrates must be bound in the molecule in a manner quite different from that of the oxygen atoms in nitrites. The writer has used the specific term "nitrate oxygen atom"<sup>7</sup> to designate that oxygen atom which

<sup>3</sup> Through cooperation with Prof. Treat B. Johnson, who has supplied rare research material, it has been possible to extend the field of investigation into the pyrimidine and purine series. The results of these researches, which are of immediate biochemical interest, will be published at a later date.

<sup>4</sup> Menaul, P., *J. Biol. Chem.*, 1921, xlv, 297.

<sup>5</sup> Baudisch, O., *Ber. chem. Ges.*, 1916, xlix, 1151.

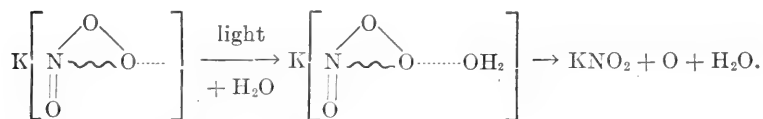
<sup>6</sup> Baudisch, O., *Ber. chem. Ges.*, 1911, xlv, 1009.

<sup>7</sup> Baudisch, O., *Ber. chem. Ges.*, 1912, xlv, 2879; 1916, xlix, 1176.



is easily split off from such salts either under the influence of light or of metallic iron. That we are dealing here with such a labile oxygen linking in nitrates is demonstrated to us by nature, in that the various nitrifying and denitrifying bacteria have the power to differentiate between nitrate and nitrite oxygen. The question naturally arises, under what influence or by means of what power an atom of oxygen can be split out of potassium nitrate, for example, with the formation of a nitrite. This problem was first attacked from a purely chemical standpoint, but it is now proposed to continue the study also from a biological point of view.

In explanation of the photochemical reduction of nitrates in aqueous solution, we may assume, according to Werner's theory<sup>8</sup> of reaction, an activation of the residual valence of an oxygen atom of the nitrate and of the oxygen atom of the water, resulting in the attraction of molecules of water into the inner sphere of the nitrate molecule. There follows a dissociation of the nitrate molecule with the formation of oxygen as is expressed by the following equation:

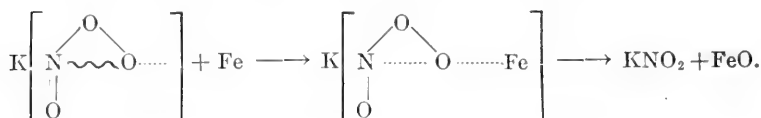


In other words, this reduction process takes place under the influence of light even in an atmosphere of oxygen, and stops at the nitrite stage. The reverse process can also take place and nitrite is readily formed from ammonia by photochemical oxidation without, however, any production of nitrate.

It is apparent from these facts that light can readily split off an atom of oxygen from nitrates of the alkali metals, without the presence or influence of either nascent or molecular hydrogen. This dissociation of nitrate into oxygen and nitrite can also be brought about by means of metallic iron as well as under the influence of the energy of light. If a neutral oxygen-free solution of potassium nitrate be shaken in a vacuum with active iron prepared by reduction with hydrogen, the supernatant liquor obtained

<sup>8</sup> Werner, A., *Neuere Anschauungen auf dem Gebiete der anorganische Chemie*, Brunswick, 4th edition, 1920.

after the iron powder has been allowed to settle will give every reaction applicable for the detection of nitrous acid.<sup>9</sup> In other words, metallic iron will easily reduce potassium nitrate to potassium nitrite in the cold in the absence of every trace of oxygen, and under conditions such that neither the action on the iron by water nor the effect of nascent hydrogen can possibly come into consideration. These results lead to the assumption, therefore, that iron readily splits off an oxygen atom of the nitrate after having first entered into a loose combination with it. This change may be expressed by the following equation:



From these examples it is seen that the photochemical reduction of nitrates to nitrites and their reduction by means of metallic iron are similar in nature, and in both cases there occurs either an activation or mobilization of the valence energy leading to the formation of an unstable addition product, which finally breaks down into the final products of reaction. Although it has been possible to show a relation between the photochemical reduction of nitrates to nitrites and the corresponding biological reduction in green leaves, it has not yet been possible to connect known chemical reduction processes with the biological reductions occurring naturally in bacteria or in molds.

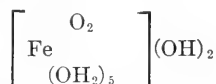
It formerly appeared scarcely possible to attach any biological importance to ferrous hydroxide in these reduction processes because, as was commonly believed, the reaction proceeded stoichiometrically and ferric hydroxide was formed at the expense of the oxygen split off from the nitrate. In biological processes, however, one cannot speak of stoichiometrical reactions in connection with metals, because, as is well known, they are present only in traces. Their action must be explained, on the contrary, by a particular energy inherent in their molecules, and consequently the writer has been accustomed to consider the metals as functioning in biological processes either as "mobilizers" or "catalysts,"

<sup>9</sup> Baudisch, O., *Ber. chem. Ges.*, 1921, liv, 406.

having the power to bring into play the latent energy of certain organic molecules.

From the point of view of Werner's coordination theory, it is possible to conceive of a relation between the mobilizing power of such a catalytic agent, and the peculiar power expressed by residual valency, which, as is well known, has the ability to draw new atoms, molecules, and radicals into the sphere of action of the internal nucleus of the metal, resulting in the most varied types of reactions. In fact, it is well known that extremely finely divided metals, such as platinum, palladium, or iron, possess these valence powers to a large degree, and their specific action has often been placed in parallel with purely biological processes.

The reduction of nitrates with ferrous hydroxide assumed a new interest when it was discovered that this reagent alone does not split off nitrate oxygen as had been assumed, but reduces nitrates only under the influence of oxygen.<sup>10</sup> As it is well known that white ferrous hydroxide is converted instantaneously into green ferrous hydroxide peroxide by the oxygen of the air, it is reasonable to say, therefore, that this polymolecular combination or peroxide is the active reagent which brings about this transformation of nitrates into nitrites.



(Coordination formula for ferrous hydroxide peroxide.)

The mechanism of this reduction has not yet been explained, but the attempt will be made here to show that the free energy of ferrous hydroxide is increased enormously by a loose combination with an oxygen molecule, and that this increase in energy makes itself apparent both physically and chemically. The striking effect on the color of white ferrous hydroxide, which is caused by the smallest trace of oxygen, shows that the oxygen enters into the inner sphere of the iron nucleus. Schäfer<sup>11</sup> has proved spectroscopically that such extraordinary alterations in color, either in the visible or in the invisible part of the spectrum, can only take place simultaneously with changes in the inner sphere

<sup>10</sup> Baudisch, O., *Ber. chem. Ges.*, 1921, liv, 410.

<sup>11</sup> Schäfer, K., *Z. anorg. Chem.*, 1918, lxxxvi, 221.

of the molecule. It has, however, not yet been shown how many oxygen molecules are present in such a molecular combination. It may be possible that the observation of Meyer,<sup>12</sup> who discovered that strongly magnetic substances were rich in absorption bands, whereas diamagnetic substances were poor in absorption bands, has some connection with these facts. This coincides completely with the action of the above mentioned iron compound, because, while the white ferrous hydroxide has practically no magnetic properties, the green to black ferrous hydroxide peroxide possesses magnetic properties which are almost equal to that of metallic iron. According to Hilpert,<sup>13</sup> when a stream of air or oxygen is led through a precipitate of ferrous hydroxide, the magnetic properties of the precipitate increase rapidly. Quartaroli<sup>14</sup> has also shown that oxidation with air converts the ordinary non-magnetic ferrous hydroxide into mixed ferro-ferri oxides, which possess a susceptibility almost a hundred times greater than ferric salts. In fact the magnetic susceptibility of  $\text{Fe}_3\text{O}_4$  approaches that of the metal iron itself. It may be concluded, therefore, from our present knowledge, that there is a very close relationship between the physical properties of metallic iron and those of ferrous hydroxide peroxide, and it will be of the greatest interest and importance to determine whether there is also any direct relationship between the peculiar chemical properties of this peroxide and those of finely divided metals. The simplest explanation, therefore, for the reduction of nitrates is the assumption that freshly precipitated, colloidal ferrous hydroxide peroxide acts catalytically as a finely divided metal.

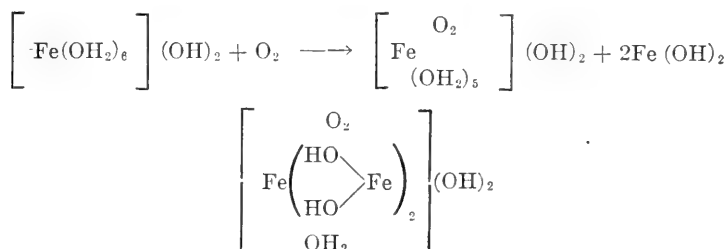
It seems very probable that the peculiar properties resulting from the colloidal nature of ferrous hydroxide peroxide and the properties of the metal resulting from its position as the central atom of a complex system actually coalesce. Furthermore, these characteristic properties apply only to the peroxide and not to ferrous hydroxide, because the latter compound is not only unable to bring about a reduction of nitrates, but also will not react to form polynuclear compounds. Not until brought under the influence of oxygen does the central iron nucleus of ferrous hy-

<sup>12</sup> Meyer, S., *Wied. Ann.*, 1899, lxxviii, 325.

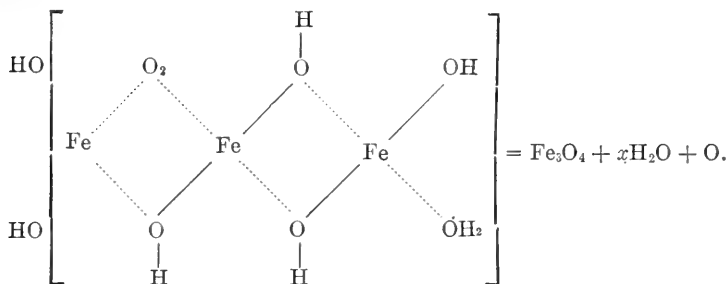
<sup>13</sup> Hilpert, S., *Ber. chem. Ges.*, 1909, xlii, 2248.

<sup>14</sup> Quartaroli, A., *Chem. Zentr.*, 1917, i, 729.

dioxide or other ferrous salts acquire the property of attracting new molecules of ferrous hydroxide into its inner sphere. This coordination combination exists, according to Werner, not between iron and iron, but between the active hydroxyl oxygen atoms of the ferrous hydroxide, which are attracted to the central iron nucleus of the peroxide by its residual valency. It is also possible for the iron atoms of ferrous hydroxide to be held in combination through the peroxide oxygen atoms, as has been demonstrated in the case of cobalt compounds by the classical researches of Werner. The mechanism of the autooxidation of ferrous hydroxide and the formation of strongly magnetic  $\text{Fe}_3\text{O}_4 \cdot x\text{H}_2\text{O}$  may be expressed by the following formulas:



The structure of this polynuclear combination may be expressed graphically as follows:



From these graphic representations it is seen that ferrous hydroxide is converted by the absorption of oxygen into a peroxide of greater potential energy, whose iron nucleus, as experience has shown, possesses the property of intensifying the activity of and of entering into loose combination with the residual valence of oxygen atoms in other molecules of ferrous hydroxide present.

With the coordination theory of Werner as a basis, it becomes apparent from the foregoing that ferrous hydroxide may be transformed by the absorption of oxygen into a complex salt whose iron nucleus, just as finely divided metals, may enter into a wide range of reactions. A nitrate oxygen atom may be split off as well by means of light as by means of metallic iron or by ferrous hydroxide peroxide. All three of these processes of reduction may be considered to depend upon the same principle; namely, the mobilization or activation of the energy in the residual valence of the reacting materials. Ferrous hydroxide peroxide reacts most probably in a very similar manner to finely divided iron or platinum.

*The Reduction of Nitrites.*

Aqueous solutions of potassium nitrite containing easily oxidizable substances, such as alcohols, aldehydes, sugars, starches, etc., suffer a comparatively rapid reduction and decomposition under the influence of diffused daylight, and the change may be expressed very simply as follows:



The presence of potassium nitrosyl in solution may be detected by means of its condensation reaction with aldehydes (Angeli's aldehyde reaction).<sup>15</sup> Hydroxamic acids are formed as products of reaction, and as is well known, these acids give characteristic complex salts with iron which are colored a deep reddish violet. This reduction of potassium nitrite to potassium nitrosyl can also be accomplished by means of complex iron salts. The smooth reduction of potassium nitrite *via* potassium nitrosyl to ammonia by means of glucose, in the presence of very small quantities of iron, possesses particular biological interest. The system, glucose + iron + alkali, which is a fundamentally new reducing combination, does not attack in the least the alkali salts of nitric acid.<sup>16</sup> It is therefore possible to make a quantitative separation

<sup>15</sup> Angeli, A., *Samml. Chem. u. chem. Techn. Vortr.*, 1908, xiii.

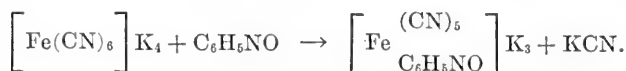
<sup>16</sup> A method, based on this observation, has been developed for the quantitative determination of nitrites and nitrates in the presence of other nitrogen compounds in soil extracts. Pfeiffer, T., and Simmerbacher, W., *Land. Versuchsstat.*, 1916, xciii, 65; *J. Soc. Chem. Ind.*, 1919, xxxviii, 507.

between nitrate and nitrite by means of a grape sugar-iron-alkali solution. Grape sugar, which is absolutely free from iron, does not cause the slightest reduction, even on heating, of nitrites; and also chrysarobin (1,8-dioxy-3-methyl-anthranol), which occurs quite widely in the vegetable kingdom, has not the ability of reducing the salts of nitrous acid. On the other hand, the addition of traces of any iron salt to an alkaline solution of either of these substances enables them to reduce immediately the nitrites by way of nitrosyl to free ammonia. The particular part that iron plays in these reactions remained a mystery for a long time, but seems now to have been explained quite satisfactorily.

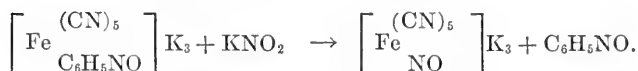
To explain this reduction process and its application for the quantitative separation of nitrates and nitrites, we may assume that the unsaturated, trivalent nitrogen atom of the nitrite molecule enters, through its activated residual valency, into a loose combination with the central iron nucleus of whatever complex salt is present, and then dissociates through the intermediate formation of nitrosyl into NO and K. The residual valency of the nitrate oxygen atom is not sufficiently active or powerful to displace the molecules or radicals already present in the inner sphere of the iron nucleus, and therefore these compounds are not attacked. That these reductions may all be considered as complex salt reactions, or in other words "nuclear exchange or displacement reactions" may be shown by the following example: A solution of 1 gm. of  $K_4Fe(CN)_6$ , 1 gm. of  $NaNO_2$ , and 5 gm. of sodium carbonate in 200 cc. of water, is distilled in a stream of oxygen. After interrupting the stream of oxygen, it is possible to detect nitrous acid in the distillate. The oxygen, under the influence of heat, has displaced the cyanogen group from the inner sphere, and in its place a molecule of nitrite has entered. The nitrite, however, decomposes and its scission product, NO, which at first takes its place in the inner sphere of the iron nucleus, is in turn displaced by the oxygen, and finally passes over into the distillate where it is easily detected as nitrous acid.

The fact that this remarkable reaction may not only be influenced by daylight, but in some cases will not take place except under the influence of daylight, is of particular chemical and biological interest. For example, if a freshly prepared solution of potassium ferrocyanide be treated with an aqueous alcohol

solution of nitrosobenzene and placed under the influence of diffused daylight, the solution which in the beginning possesses a weak, greenish yellow color, changes in a few minutes to a deep reddish violet. The mechanism of the reaction may be expressed as follows:



On treating the aqueous solution of this reddish violet compound with an excess of potassium nitrite and again placing the solution under the influence of diffused daylight the following decompositions take place:



The deep violet color of the solution disappears very rapidly and the reddish yellow or potassium nitroprusside takes its place. By means of this reaction, the reduction of sodium nitrite by means of a complex iron salt and light is demonstrated.

These processes of reduction of nitrites by way of nitrosyl to ammonia, may be drawn into intimate relation with biological reductions of nitrite, particularly as sugar, or its products of decomposition, and iron constantly accompany the nitrates in plants or in bacteria. Kostyschew and Tsweskowa<sup>17</sup> state that the reduction of nitrate to nitrous acid takes place without the presence of any sugar, but that the further conversion of the nitrous acid, at least in the case of *Mucor racemosus*, is accomplished only in the presence of sugar. It seems likely, from investigations with cholera bacteria, that nitrates are reduced to nitrites by way of nitrosyl, because it was possible to detect the alkaline decomposition products of nitrosyl, for example, NO and NH<sub>3</sub> (the latter as a reduction product of NO), in the volatile portions of alkaline cholera peptone cultures.<sup>18</sup>

<sup>17</sup> Kostyschew, S., and Tsweskowa, E., *Z. physiol. Chem.*, 1920, cxv, 171.

<sup>18</sup> Baudisch, O., *Ber. chem. Ges.*, 1916, xlix, 1148.



The assumption that nitrite is converted into nitrosyl finds a further support in the qualitative and quantitative composition of the gases which are produced during the photochemical reduction of nitrites in the presence of formaldehyde and during the biological reduction. For example, those bacteria which have the property of decomposing nitrates, produce a fermentation gas which consists of about 65 to 72 per cent  $N_2O$ .<sup>5</sup> In addition to the nitrous oxide, there is also always formed a little nitrogen, traces of NO, and also of prussic acid. A formaldehyde-potassium nitrite solution produces a gas under the influence of diffused daylight, which contains 64 per cent  $N_2O$ , as well as very small quantities of NO and HCN. The latter two gases were detected qualitatively by sensitive reactions. Clawson and Young<sup>19</sup> have detected prussic acid in cultures of *Bacillus pyocyaneus* and of other bacteria.

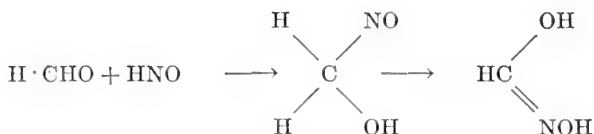
To summarize, the photochemical reduction of the alkaline nitrites proceeds by way of the reactive intermediate product, potassium nitrosyl, which may be detected by means of aldehydes, as well in the case of the photochemical reduction of nitrites as in the case of a reduction with the system, grape sugar + iron + alkali. Certain complex iron salts possess the property of reducing nitrites, whereas under these conditions nitrates remain unchanged. The reduction of the alkali nitrites by means of complex iron salts depends most likely upon the residual valency of the central iron nucleus and in all these changes light as well as heat exercises a very fundamental influence.

*Synthesis of Organic Compounds Containing Nitrogen from Inorganic Compounds of Nitrogen.*

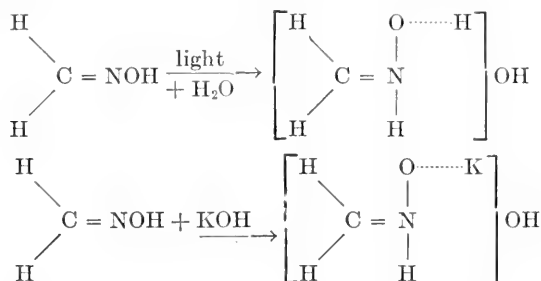
Nitrosyl, which is formed in the reduction of alkali nitrates, interacts readily with formaldehyde with formation of formhydroxamic acid (Angeli's aldehyde reaction). This reaction proceeds, as was shown by the writer and Coert,<sup>20</sup> through the intermediate formation of nitroso methyl alcohol. Formhydroxamic acid is then formed from this by molecular rearrangement, and may be detected easily by means of its characteristic iron and copper salts, which are highly colored.

<sup>19</sup> Clawson, B. J., and Young, C. C., *J. Biol. Chem.*, 1913, xv, 419.

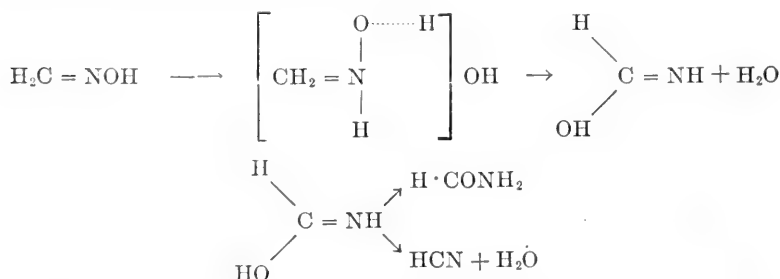
<sup>20</sup> Baudisch, O., and Coert, J. H., *Ber. chem. Ges.*, 1912, xlv, 1775.



Formhydroxamic acid, under the influence of light, loses an atom of oxygen and is converted into formaldoxime, which is also characterized by its great reactivity. The stable form of this compound is altered under the influence of light and also alkali, and is transformed into an extremely labile modification. This transformation may be expressed as follows:<sup>21</sup>

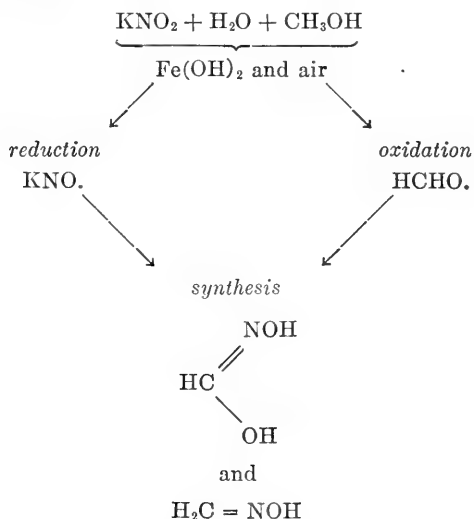


As is well known, formaldoxime exhibits a strong tendency to polymerize with the formation of three-carbon chain compounds. Its labile form is also capable of reacting further with aldehydes with the formation of three-carbon compounds, and furthermore under the influence of light combines with formaldehyde to form cyclic combinations containing both nitrogen and carbon. Under the influence of light formaldoxime undergoes, in part, a Beckmann rearrangement with formation of formamide, and, in part, a complete dissociation into prussic acid, water, and ammonia. These changes are expressed below:



<sup>21</sup> Baudisch, O., *Ber. chem. Ges.*, 1916, xlix, 1159.

The small quantities of prussic acid that are always found accompanying the treatment of formaldehyde in nitrate solutions with light, or by the reduction of nitrates with bacteria, may have been formed in accordance with the above reaction from the aldoximes. The photochemical formation of nitroso methyl alcohol (or hydroxamic acid) from formaldehyde, methyl alcohol, and nitrosyl may be the chemical counterpart of a possible biochemical formation of carbon-nitrogen-containing organic substances from inorganic nitrogen. The next step to amino nitrogen is simpler and may either take place by reduction or, as in the case of aldoxime, by simple rearrangement. It seems probable that the proof of the biological importance of nitroso methyl alcohol or formhydroxamic acid is found in its marked reactivity and in the pronounced tendency which it has to rearrangement, to polymerization, and to the formation of complex salts, particularly of iron. It seems extremely possible to introduce amino nitrogen into the higher alcohols, sugars, starches, etc., by means of nitrosyl which is formed photochemically from the nitrites and which is capable of entering into such widely different reactions.



Recent work<sup>10</sup> has revealed the fact that light may be replaced in certain cases by means of ferrous hydroxide and oxygen. Fer-

rous hydroxide peroxide oxidizes alcohols to aldehydes simultaneously with the reduction of nitrites to nitrosyl, and therefore synthesis of formhydroxamic acid or formaldoxime takes place. In fact, in these solutions of ferrous sulfate, containing bicarbonate and nitrite, three dissimilar reactions proceed simultaneously—oxidation, reduction, and synthesis.

To summarize, nitrosyl is formed from alkali nitrites photochemically and by reduction with glucose in the presence of iron and by reduction with ferrous hydroxide in the presence of oxygen. The formation of carbon and nitrogen organic compounds in green plants and bacterial cultures from inorganic nitrogen, and the production of  $N_2O$ ,  $N_2$ ,  $NO$ , and  $HCN$  during fermentation and photochemical reduction may be explained by the intermediate formation of nitrosyl  $H\{NO^{22}$  and its subsequent reaction with aldehydic combinations.

<sup>22</sup> Bracket indicates labile character.

## STUDIES ON THE PHYSIOLOGICAL ACTION OF SOME PROTEIN DERIVATIVES.

### VII. THE INFLUENCE OF VARIOUS PROTEIN SPLIT PRODUCTS ON THE METABOLISM OF FASTING DOGS.

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New Haven.)*

(Received for publication, August 1, 1921.)

It is the design of this study to extend certain recently developed aspects of the problem of proteose intoxication.

The toxic properties of protein split products, at the proteose stage of hydrolysis, have called forth a wide literature (1). This has been primarily concerned with the clinical effects of a rapid intravenous injection of a concentrated solution of proteose. The chief signs emphasized were, the depression of blood pressure and respiration, the delayed coagulability of the blood, the lymphagoguic effect, the constitutional depression or "peptone shock," the gastrointestinal irritation, the diminished response to subsequent injections or "immunity," the leucopenia, the marked concentration of the blood,<sup>1</sup> the severe acidosis,<sup>1</sup> and the rapidly fatal issue. These constitute a syndrome rivalling in violence any that we know. Naturally proteoses were assigned the toxic agency in diseases where injury of tissue was a factor. In the absence of a more tangible cause it was widely assumed that "the absorption of toxic proteoses" explained an acute intoxication. Recently, these assumptions have been given experimental ground.

Vaughan (2) and his coworkers have prepared toxic split products from a variety of proteins possessing many of the properties of proteoses. They concluded from their experiments that all

<sup>1</sup> Author's unpublished data.

proteins contain a toxic nucleus which when released gives rise to an intoxication identical with an infectious process. They have offered an attractive theory of infection and immunity on the basis of this work.

Biedl and Kraus (3) showed the physiological resemblance between peptone shock<sup>2</sup> and anaphylactic shock, and urged the view that the formation of digestion products identical with those present in the market peptone were responsible for anaphylactic shock. Indeed the mechanism of anaphylactic shock as presented in the work of Jobling, Peterson, and Eggstein (4) is essentially a proteose intoxication, by proteose derived from the host's serum as a result of the disturbance of the ferment antiferment balance. And more to our point Whipple and Cooke (5) and their coworkers in a series of extensive experiments have demonstrated that the intoxication of intestinal obstruction in dogs is due to the absorption of a toxic proteose produced in the obstructed gut. They have isolated and purified a proteose and clinically reproduced the disease by injection of the substance in normal dogs. Moreover they have shown that such injections cause a very large increase in the output of urinary nitrogen and a significant rise in non-protein nitrogen of the blood.

But they have gone further and it is with this phase that the present work is concerned. In a series of papers on proteose intoxications and injury of body protein (6) these authors showed that inflammatory processes initiated either through bacterial agency or by aseptic means also call forth an increase in urinary nitrogen excretion and blood non-protein nitrogen. Hence they offer the suggestion that perhaps every inflammatory process is fundamentally a proteose intoxication. According to this view an inflammatory process may so injure body cells that toxic proteoses are formed which injure other cells, etc., so that a vicious circle is established. This hypothesis is certainly highly suggestive and if correct would render all inflammatory reactions simple of explanation and reduce the whole process to a single simple reaction.

We have been working for some time on proteose intoxication and have been particularly interested in these view-points. We have asked ourselves first of all whether the catabolic reactions called forth by Whipple's toxic proteose are specific or whether

<sup>2</sup> Referring to commercial "Witte pepton" composed of a mixture of proteoses and peptone.

such reactions are common to all proteoses. And, secondly, are there any other protein split products that are likely to occur in tissue injury capable of the same catabolic effect?

### *Technique.*

The plan of the experiments was to follow the urinary nitrogen, creatine, and phosphorus in fasting dogs until a nitrogen level had been reached, to inject intravenously a solution of the substance under consideration in normal saline solution, then to continue the experiment until these excretory products had returned to normal. Usually 4 days of fasting sufficed to bring the dogs to a basal nitrogen level. On the 4th day the injection was made into the jugular vein, under light ether anesthesia. The rate of injection was usually slow enough to avoid acute shock (in 5 to 15 minutes). The preparations will be described in their places.

The animals were kept in standard metabolism cages and the urine collected by catheterization every 24 hours. They were allowed to drink water according to their desire. The dogs were usually house-broken so that we had little trouble from contaminations of the urine with feces or vomitus. When such occurred, they were included in the 24 hour samples. Nitrogen was done by the Kjeldahl method, creatine and creatinine by Folin's methods, and total phosphorus by the uranium acetate method. We confined ourselves to these urinary constituents because we found early that they were the only ones to show any significant change. The clinical effects were typical and are not recorded because they have been sufficiently described elsewhere.

### *Control Experiments.*

In Table I and Chart 1 are given the results of several control experiments. Dog 1 was fasted for 7 days. It will be observed the constituents maintain a comparable level. Dog 2 received an intravenous injection of normal saline solution under identical conditions with the other experiments. The technique employed apparently has no influence on the course of metabolism. In Dogs 14, 15, and 16 we tried to show that the injection of a colloidal solution was in itself unable to affect

metabolism. This was of importance, because most of the substances subsequently injected were colloidal in character. The

TABLE I.

*Influence of Fasting, Injection of Normal Saline Solution, and Non-Protein Colloids.*

Dog.	Day.	Weight.	Urine volume.	Total N.	Crea- tine.	P <sub>2</sub> O <sub>5</sub>	Remarks.
		<i>kg.</i>	<i>cc.</i>	<i>gm.</i>	<i>mg.</i>	<i>mg.</i>	
1	2		90	1.890	20	350	Fasting only.
	3		80	1.830	35	362	
	4		70	1.830	49	400	
	5		73	1.850	62	387	
	6		88	1.710	54	312	
	7		72	1.740	40	375	
2	5		66	3.020	29	340	Injected 50 cc. of normal saline solution.
	6		57	2.880	35	300	
	7	8.5	60	3.030	26	359	
	8		56	2.920	14	347	
	9		54	2.780	14	385	
	10		53	2.710	30	341	
14	3	15.0	74	3.380	45	490	Injected 50 cc. of solution of soluble starch.
	4		90	3.250	51	580	
	5		78	3.040	29	440	
	6		74	3.250	50	438	
	7		72	2.960	22	420	
15	4		110	3.620	102	663	Injected 30 cc. of strong solution of inulin.
	5	10.4	120	3.330	117	675	
	6		90	2.840	81	525	
	7		100	2.640	83	565	
16	4	7.8	90	2.950	20	338	Injected 50 cc. of 6 per cent gum acacia solution.
	5		92	2.205	4	337	
	6		96	1.890	11	312	
	7		92	1.890	18	262	
	8		72	1.830			

influence of the physical state of the solution injected is negative, from our view-point.



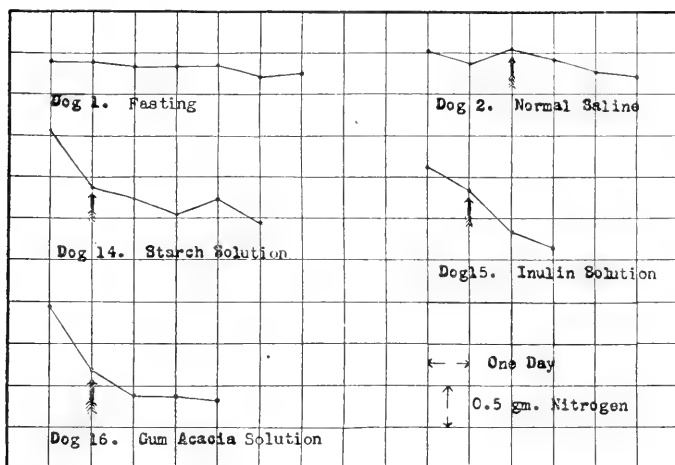


CHART 1. Control experiments. Influence of fasting, the injection of normal saline solution, and of non-protein colloids, on the daily excretion of nitrogen. Arrows show time of injection.

#### *Effect of Amino-Acids, Amines, and in Vitro Autolysates.*

At various times it has been claimed that specific amino-acids are responsible for the toxic effects of protein split products (7). This contention has been disposed of as far as the clinical effects are concerned (1). What is the influence of free amino-acids on tissue catabolism?

In Table II and Chart 2 are given the results of the injection of various free amino-acids and mixtures. Dogs 17, 18, and 19 received pure amino-acids, leucine, glycocoll, and alanine. The results are entirely negative. The amount of nitrogen injected is excreted, but no more. In Dog 18 the creatine showed a marked rise, but this effect could not be repeated in Dog 19. We may therefore disregard it. Dogs 20 and 21 received a mixture of amino-acids prepared from the hydrolysis of casein until biuret-free. These preparations contained most of the important amino-acids yet were entirely innocuous. It might be argued that the responsible amino-acid was not present in sufficient concentration. But it will be seen that one-half the amount of nitrogen here injected, also derived from casein but given in the form of a pro-

TABLE II.

*Influence of Amino-Acids, Histamine, and in Vitro Autolysate.*

Dog.	Day.	Weight.	Urine volume.	Total N.	Crea- tine.	P <sub>2</sub> O <sub>5</sub>	Remarks.
		<i>kg.</i>	<i>cc.</i>	<i>gm.</i>	<i>mg.</i>	<i>mg.</i>	
17	2	9.6	72	2.330	72	400	Injected 50 cc. solution of glycocoll (0.288 gm. N).
	3		44	2.150	26	345	
	4		70	2.240	24	430	
	5	9.0	34	1.400	0	240	Injected 50 cc. solution of leucine (0.270 gm. N).
	6		40	1.692	25	240	
	7		48	1.746	0	220	
	8		41	1.960	25	220	
	9		50	1.600	7	230	
18	2	12.7	90	2.272	80	440	Injected 100 cc. solution of alanine (0.75 gm. N).
	3		68	1.892	0	500	
	4		130	2.552	8	520	
	5		60	2.192	115	420	
	6		54	1.888	110	380	
19	3	12.9	80	2.920	16	460	Injected 110 cc. solution of alanine (1.87 gm. N).
	4		76	3.032	8	490	
	5		172	4.600	0	540	
	6		80	2.520	27	340	
	7		90	2.620	3	370	
20	3	10.5	82	2.950	15	775	Injected 65 cc. rapidly, biuret-free casein diges- tion product (0.665 gm. N).
	4		72	2.760	24	550	
	5		114	3.720	14	600	
	6		86	2.910	28	700	
	7		100	3.150			
21	4	7.1	99	2.850	135	638	Injected 80 cc. of mixture of amino-acids (biuret- free casein digest, 0.489 gm. N).
	5		96	2.820	193	812	
	6		104	3.000	115	537	
	7		93	2.475	90	425	

TABLE II—*Continued.*

Dog.	Day.	Weight.	Urine volume.	Total N.	Crea- tine.	P <sub>2</sub> O <sub>5</sub>	Remarks.
		<i>kg.</i>	<i>cc.</i>	<i>gm.</i>	<i>mg.</i>	<i>mg.</i>	
22	2		148	3.704	42	730	
	3	16.8	100	3.424	0	720	
	4		130	3.160	87	720	Injected 0.5 mg. of histamine per kilo.
	5	16.22	84	3.000	12	600	
	6		114	2.880	25	740	Injected 1 mg. of histamine per kilo.
	7		92	2.736	0	420	
39	3		65	1.680	69	340	
	4	6.7	55	1.560	40	280	
	5		345	1.320	78	425	Injected 1 mg. of histamine per kilo.
	6		94	1.440	82	180	
27	3		90	1.995	76	400	
	4	6.7	80	1.860	73	308	
	5		440	2.250	107	400	Injected 15 cc. of dog muscle autolysate (0.432 gm. N). Coagulable proteins absent. Proteose trace.
	6		124	1.800	90	175	
28	4	10.5	205	2.580	25	440	
	5		615	4.850	258	975	Injected 70 cc. of dog muscle autolysate (1.95 gm. N). Coagulable proteins absent. Proteose trace.
	6		253	2.780	178	320	

teose, was extremely destructive of tissue (Dog 24, Chart 3 and Table III). Hence, if any particular amino-acid is the toxic agent it had opportunity to assert itself. We may conclude therefore that the amino-acids themselves are incapable of producing destruction of tissue and are not contributing factors in this respect to the toxicity of proteoses.

Recently Dale and coworkers (8) demonstrated the similarity of histamine shock and peptone shock, and Abel and Kubota (9)

claimed that histamine is the responsible agent in the toxicity of Witte peptone. Does histamine, one of the most toxic of the amines, have any influence on tissue catabolism? In Dogs 22

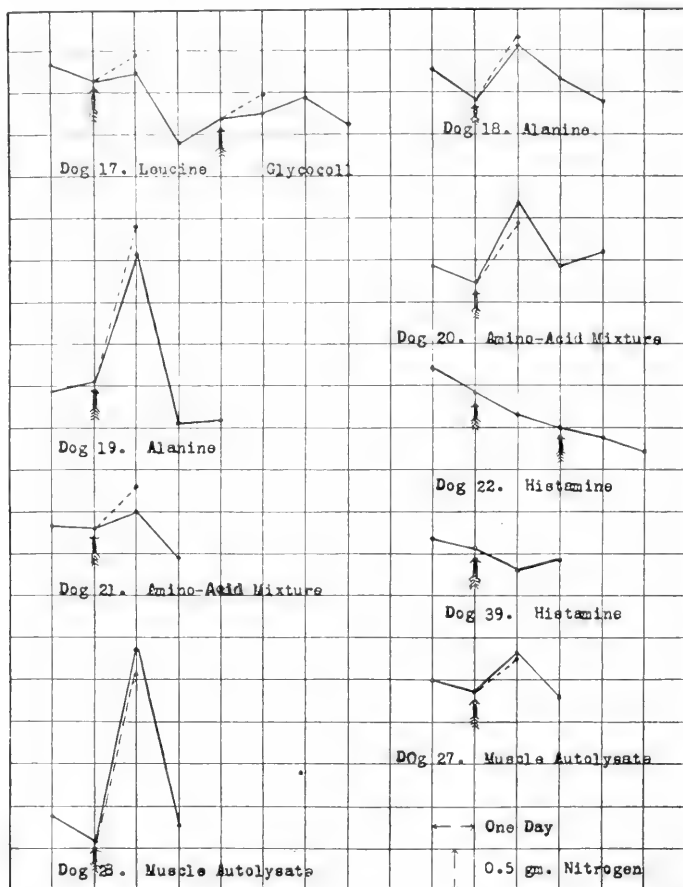


CHART 2. Influence of amino-acids and amines on the daily excretion of nitrogen. Arrows show time of injection. Dotted lines show amount of nitrogen injected.

and 39 we injected histamine in doses sufficiently large to produce shock and yet the effect on the elimination of nitrogen, creatine, and phosphates was negative. These doses (1 mg. of base per kilo) constitute about 300 times the amount of histamine contained

in a toxic dose of Witte peptone (Dog 5, Chart 3), according to the analysis of Hanke and Koessler (10). Hence the amine content of proteoses as represented by histamine cannot be the catabolic agent of proteoses. This agrees with the findings of Hanke and Koessler (10) who showed that histamine-free proteose gives the typical clinical toxic picture.

Dogs 27 and 28 show that autolysate free from coagulable protein, rich in amino-acids but poor in proteose and peptone as shown by a faint biuret reaction, produced no increase in urinary nitrogen. This substance was prepared by autolyzing 1 kilo of dog's muscle under toluene, for a month, precipitating the proteins, and concentrating the residue. Unfortunately the creatine and phosphates were not determined in the injected material. That the undoubtedly rich content of creatine and phosphates of the injected material would account for the large output of those constituents in Dog 28 which received the undiluted residue, seems to us more rational than to accept the indication that tissue is destroyed. This view agrees with the negative influence on the total nitrogen.

These last two experiments are of interest in indicating that autolysis *in vitro* and autolysis *in vivo* although probably the result of the same mechanism are capable of contrary effects. The autolysis that obtains during septic or aseptic suppuration is toxic, for these are usually accompanied by fever, depression, and as Whipple has shown by tissue catabolism. The explanation it seems to us is to be sought in the general quantitative character of the end-products which the conditions of the autolysis determine, rather than in the production of special products endowed with specific properties. Had the autolysate given to Dogs 27 and 28 contained a sufficient amount of the more complex protein derivatives like proteose, in other words had the digestion been less complete, it would undoubtedly have been toxic also as will be clear from the next series of experiments.

#### *The Influence of Ordinary Proteoses on Catabolism.*

As has been already stated, the question of prime importance is: Are the reactions called forth by Whipple's toxic proteose specific, or are they common to other proteoses? Clinically



TABLE III—*Continued.*

Dog.	Day.	Weight.	Urine volume.	Total N.	Crea- tine.	P <sub>2</sub> O <sub>5</sub>	Remarks.
		<i>kg.</i>	<i>cc.</i>	<i>gm.</i>	<i>mg.</i>	<i>mg.</i>	
23	4	11.6	120	3.020	117	587	Injected pure deuteroproteose egg albumin—0.15 gm. per kilo (0.174 gm. N).
	5		113	2.640	84	650	
	6		150	3.690	149	1,050	
	7		135	2.650	54	525	
24	4	16.3	140	3.750	135	725	Injected pure deuterocaseose—0.3 gm. per kilo (0.370 gm. N).
	5		120	3.160	60	675	
	6		365	4.710	320	1,150	
	7		390	5.730	529	800	
	8		188	3.130	31	675	
25	4	6.8	52	1.345	9	180	Injected deuteroproteose egg albumin—0.15 gm. per kilo (0.100 gm. N).
	5		40	1.470	43	307	
	6			1.430	57	450	
	7		102	2.420	41	330	
	8		90	1.300	22	230	
30	4	17.3	130	4.620	31	625	Injected 0.1 gm. per kilo Vaughan's crude soluble poison in 50 cc. of saline solution (0.167 gm. N).
	5		170	5.025	50	775	
	6		260	6.660	173	2,400	
	7		900	9.850	655	1,670	
	8		100	14.700	490	1,700	

In Table III and Chart 3 are recorded experiments along this line. Dog 5 received a large dose of Witte peptone and it will be observed in the next 2 days he excreted about 2.5 gm. of extra nitrogen. The creatine was more than doubled and the P<sub>2</sub>O<sub>5</sub> almost doubled. The significance of an increased nitrogen output has already been discussed by Whipple (5,6) and is a clear argument for tissue destruction. The marked rise in creatine is

an added indication of the same, as has long been known. During fasting, the involution of the muscular uterus, muscular atrophy,

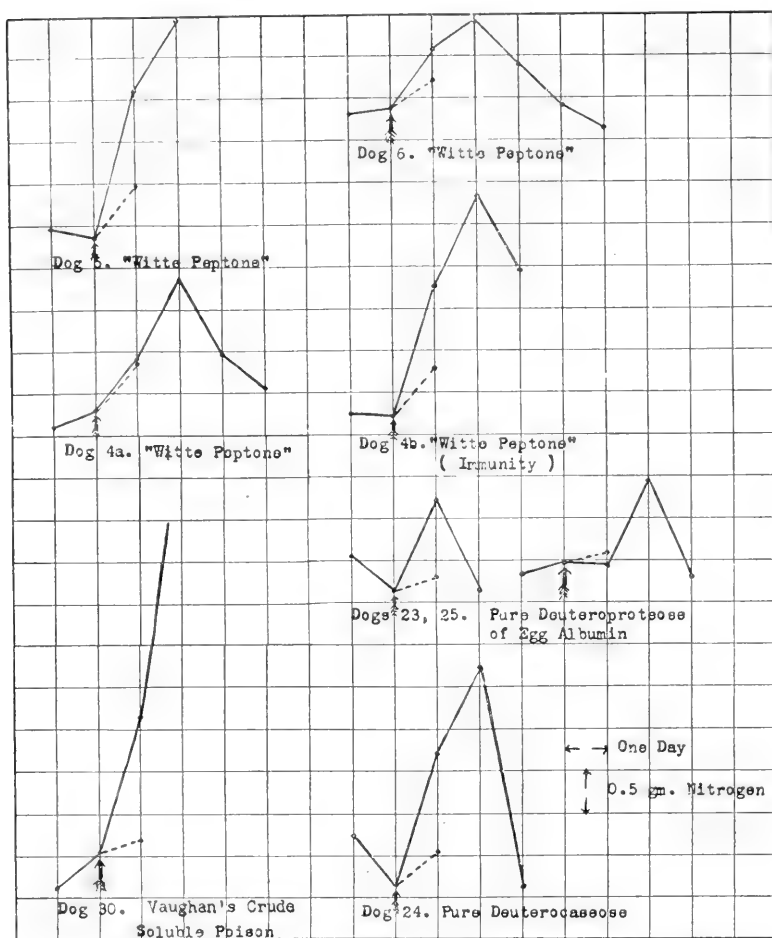


CHART 3. Influence of proteoses on daily excretion of nitrogen. Arrows show time of injection. Dotted lines show amount of nitrogen injected.

or wherever else muscle tissue is disintegrated extra creatine appears in the urine. But more than a confirmation of the evidence that the extra nitrogen gives, the large creatine output



points to the seat of the catabolism, a question that Whipple raised but could not answer from his experiments; namely, the muscle tissue.

The large output of extra phosphorus is an added indication of tissue catabolism and probably of muscle in particular. Forbes and Keith (11) in a review of the literature on this subject bring out the facts that the destruction of phosphorus-containing proteins as in general catabolism, violent exercise, the destruction of leucocytes, cancer, tuberculosis, septic conditions, fever, and acidosis, results in an excess output of phosphates. In peptone shock we have an acidosis but in these dogs shock was avoided by slow injection, hence it must be the tissue destruction factor which accounts for the extra phosphate. Since the other conditions mentioned are largely muscle wasters, the extra phosphate output here may be considered an index of muscular catabolism.

Dog 6 received half the dose of Witte peptone of Dog 5. The response in the three directions indicated is not as marked but it is definite.

Dog 4a received a dose intermediate between Dogs 5 and 6 and the response was intermediate by all three criteria. We may say that the size of the dose is an important factor in the severity of the catabolism induced. This has long been known with regard to the clinical effects, small doses being innocuous and larger doses fatal.

There is no way of accurately comparing the degree of response of our dogs with Whipple's because this author does not state his dosage in terms of nitrogen so that judgment can be made of the amount of proteose given. On the whole, our effects are neither as great nor as prolonged, but certainly of the same kind.

Dog 4b received the same dose a second time several weeks later to test for immunity but the response was much greater the second time. This may have been due to the rapidity of the injection which we know from the clinical reactions is an important factor. All three criteria of tissue catabolism are strongly present.

In order to controvert the idea that Witte peptone is an impure proteose and therefore not a fair criterion of what other proteoses will do, to Dogs 23, 24, and 25 we gave pure proteoses prepared from egg white and casein.<sup>3</sup> The response of Dogs 23 and 24

<sup>3</sup> The material formed from egg white and casein was prepared by pepsin digestion then saturated with ammonium sulfate after neutralization

although characteristic was not marked. This can be accounted for by the small dose, 0.15 gm. per kilo, which was necessitated by the fact that larger doses killed several dogs from the acute effects (blood pressure depression, etc). Dog 24 received 0.3 gm. per kilo at the usual rate and the tissue catabolism was as great as with Witte peptone. Hence the purity or impurity is of small moment so long as there is sufficient proteose present.

It will be observed in our dogs that the excess nitrogen excretion is partially delayed to the 2nd day as with Whipple's dogs. That is to say, the crest of the excretion comes on the 2nd day in most of the cases. Another point of identity is the diuresis, although that is frequently not marked.

As evidence of how much more toxic than ordinary proteoses, other protein split products can be, there is the enormous catabolic influence of Vaughan's crude soluble poison. Dog 39 received only 0.1 gm. per kilo. Witte peptone would have been harmless in that dosage. On the 2nd day the nitrogen output was doubled and on the 3rd day trebled. The creatine and phosphate kept pace. What are we to say of the relative toxicity of proteoses? It is true Vaughan's crude soluble poison is not strictly a proteose but it is not far removed from one. It gives the clinical reactions of proteoses, but more intensely. It is partially precipitated by saturation with ammonium sulfate, both filtrate and precipitate being active. It is nearer the protein end of the hydrolytic chain. But proteins as we shall see later although catabolic agents are not nearly as destructive as this substance or even Whipple's toxic proteoses. So that it cannot be the position in the hydrolytic scale that is the whole story. It seems to us reasonable to conclude that probably all true proteoses are catabolic agents, although there is considerable variability in the degree of injury induced.

of the digestion mixture and removal of undigested residue and neutralization precipitate. The proteoses were dissolved in  $H_2O$  and dialyzed free from  $(NH_4)_2SO_4$ . The mixture was filtered from the small precipitate of heteroproteose and the filtrate evaporated and saturated with NaCl which precipitated the so called protoproteose. Treatment of the filtrate from the NaCl saturation with acetic acid precipitated a mixture of proto- and deuteroproteose. The filtrate from the acetic treatment was dialyzed free from NaCl and constituted the yield of deuteroproteose. The solution freed from NaCl was concentrated to small volume and treated with alcohol. The precipitate of deuteroproteoses was washed with boiling alcohol and treated with ether while hot, and ground to a fine white powder.

TABLE IV.

*Influence of Proteins.*

Dog	Day.	Weight.	Urine volume.	Total N.	Crea- tine.	P <sub>2</sub> O <sub>5</sub>	Remarks.
		<i>kg.</i>	<i>cc.</i>	<i>gm.</i>	<i>mg.</i>	<i>mg.</i>	
7	2		75	2.533	42	360	Injected 150 cc. of solution of crystallized edestin (0.234 gm. N).
	3	8.95	60	2.493	32	370	
	4		110	3.753	35	714	
	5		95	3.466	68	290	
	6		53	3.106	51	440	
	7		62	3.080	55	320	
	8		50	2.640	36	290	
10	6		100	2.760		588	Injected 40 cc. of excelsin solution (0.060 gm. N).
	7	10.9	102	2.640	78	488	
	8		200	2.925	121	662	
	9		130	2.850	152	413	
	10		92	2.520		425	
8	2		154	4.180	81	671	Injected 35 cc. of solution of egg albumin (0.480 gm. N).
	3		112	3.972	79	668	
	4	16.1	110	4.060	70	680	
	5		187	4.480	118	998	
	6		130	4.836	81	560	
	7		108	4.040	48	482	Injected 47.7 cc. of solu- tion of egg albumin (0.411 gm. N).
	8	14.9	94	3.920	40	540	
	9		115	4.260	70	720	
	10		110	5.000	42	400	
	11		130	4.146	24	680	
9	4		100	1.835	73	325	Injected 50 cc. of egg al- bumin solution (0.480- gm. N).
	5	6.7	86	1.845	108	362	
	6		148	2.344	125	700	
	7		130	4.590	323	500	
			130	3.375	177		

TABLE IV—Continued.

Dog.	Day.	Weight.	Urine volume.	Total N.	Crea- tine.	P <sub>2</sub> O <sub>5</sub>	Remarks.
		<i>kg.</i>	<i>cc.</i>	<i>gm.</i>	<i>mg.</i>	<i>mg.</i>	
11	2		104	3.980	67	700	Injected 110 cc. of gelatin solution (0.498 gm. N).
	3		125	3.440	58	780	
	4	16.8	130	3.008	0	680	
	5		400	3.320	1	925	
	6		120	3.048	3	580	
	7		90	2.912	9	480	
12	3		68	2.448	0	480	Injected rapidly 94 cc. of gelatin solution (0.580 gm. N).
	4	11.10	84	2.916	11	580	
	5		110	3.030		665	
	6		90	3.156	20	454	
	7		88	2.920		520	
13	4	7.2	90	3.030	80	575	Injected 80 cc. of gelatin solution (0.407 gm. N).
	5		120	3.360	57	700	
	6		110	3.015	46	388	
	7		95	2.580	9	375	

*Influence of Foreign Proteins.*

Although it has long been known that foreign proteins intravenously introduced produce an increased nitrogen output (12), fever (2), and other toxic symptoms, it seemed wise to repeat this type of experimentation in order to compare the degree of catabolic effect with the similar effect of protein split products. Accordingly Table IV and Chart 4 contain the records of a few experiments.

Dog 7 received a dilute solution of edestin. The influence on the nitrogen and phosphates was quite marked, but on creatine only slight. Dog 10 responded with slight extra output of nitrogen and phosphates and a marked extra output of creatine. In view of the very small dose the effect was decidedly positive. Dogs 8 and 9 received egg albumin solution. The effect is in all essentials indicative of a tissue destruction.

Dogs 11, 12, and 13, aside from the slight effect on phosphates which cannot be stressed in itself, responded indifferently to large doses of gelatin solution. This failure to behave like other proteins is not contradictory to the general influence of proteins.

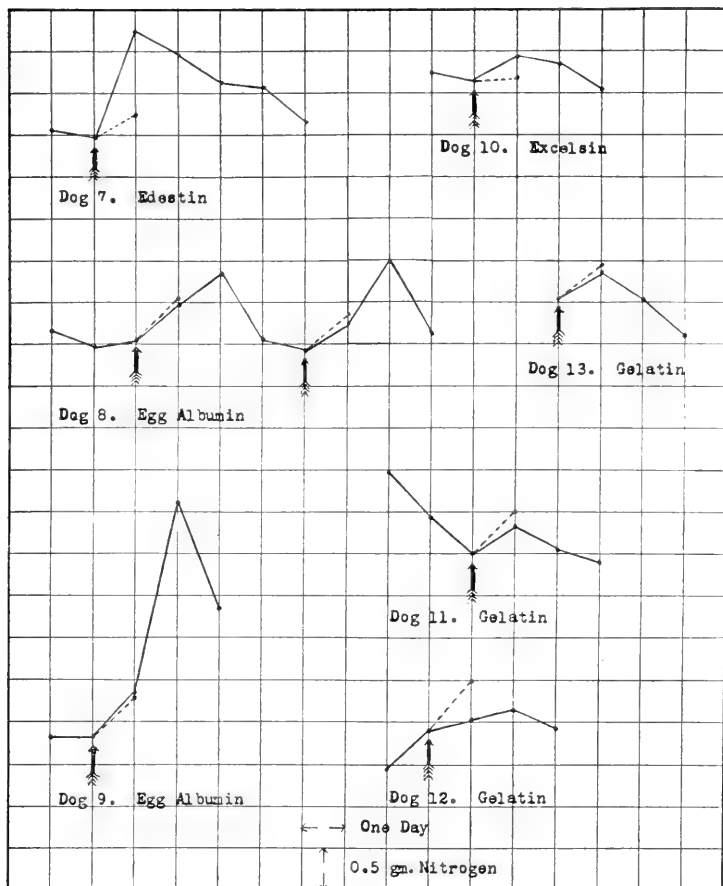


CHART 4. Influence of proteins on daily excretion of nitrogen. Arrows show time of injection. Dotted lines show amount of nitrogen injected.

Gelatin is not anaphylactogenic (13), its gelatoses are innocuous, it is deficient in important amino-acids. It confirms, however, our view that the colloidal nature of the solution is of small moment in the effects under consideration.

On the whole although the proteins are decided catabolic agents they are so to no greater degree than the proteoses. Friedmann and Isaac have shown that even homologous sera are toxic in this respect. Vaughan suggests that this is due to the fact that the proteins have become "foreign" in the process of coagulation. The same may be held for the injury to body protein during an inflammatory process. Injured protein is foreign protein and hence is a catabolic agent. In explaining then, the cause of tissue catabolism during inflammation, the attention must not be directed to the products of the injured proteins alone, but to the parent proteins as well.

#### DISCUSSION.

We believe we have answered the first question we set out to answer, to wit, that the catabolic reactions called forth by the toxic proteose of Whipple and his coworkers, are not specific but are common to this genus of compound, although to a variable degree. Whether there are other products capable of the same effect, we have already pointed to proteins themselves, and to Vaughan's crude soluble poison. We may add in anticipation of the following paper, in which the subject is given in detail, that certain nucleic acids are also capable of this catabolic effect to a very marked degree.

#### SUMMARY.

1. Ordinary proteoses induce catabolism of tissue in fasting dogs, as evidenced by a large output of urinary nitrogen, creatine, and phosphates.
2. Both pure and impure proteoses are effective.
3. Proteoses differ in the degree of their effect. The dosage and rate of injection are factors.
4. Proteins except gelatin are also capable of this effect.
5. The amino-acids, histamine, and *in vitro* autolysates, are without influence.

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## STUDIES ON THE PHYSIOLOGICAL ACTION OF SOME PROTEIN DERIVATIVES.

### VIII. THE INFLUENCE OF NUCLEIC ACIDS ON THE METABOLISM OF FASTING DOGS.

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(Received for publication, August 1, 1921.)

Whipple and Cooke<sup>1</sup> have demonstrated that the toxic proteose recovered from obstructed intestines or isolated loops, induces a marked destruction of tissue upon injection into normal dogs, as evidenced by the large output of urinary nitrogen. In the preceding paper we have shown that other proteoses possess the same property. In view of the marked similarity in physiological behavior between proteose and nucleic acid, with regard to the blood pressure depression, delayed coagulability, increased lymph flow, etc., as described by Mendel, Underhill, and White,<sup>2</sup> it seemed opportune to study whether this resemblance can be extended to the catabolic effect under discussion.

Accordingly pursuing the same method of investigation as recorded in the preceding paper we gave fasting dogs, with a constant nitrogen output, intravenous injections of representative animal and plant nucleic acids and noted the effect on the urinary nitrogen, creatine, and phosphates. The animal nucleic acids were prepared from thymus of the calf, spleen of the steer, and spleen and pancreas of the dog, by the methods described by Jones<sup>3</sup> and Baumann.<sup>4</sup> The vegetable nucleic acid was prepared

<sup>1</sup> Whipple, G. H., and Cooke, J. V., *J. Exp. Med.*, 1917, xxv, 461.

<sup>2</sup> Mendel, L. B., Underhill, F. P., and White, B., *Am. J. Physiol.*, 1902-03, viii, 377.

<sup>3</sup> Jones, W., *Nucleic acids. Their Chemical properties and physiological conduct*, New York and London, 2nd edition, 1920, 103.

<sup>4</sup> Baumann, E. J., *Proc. Soc. Exp. Biol. and Med.*, 1919-20, xvii, 118.

from fresh brewers' yeast by the method of Baumann.<sup>5,6</sup> The substances were dissolved in normal saline solution and neutralized before injection.

*Effect of Plant Nucleic Acid.*

In Chart 1 and Table I are recorded the results of experiments with yeast nucleic acid.

Dog 32 received a standard dose slowly. The output of nitrogen, creatine, and phosphates was considerably increased over the basal level. Dog 33 received the same dose rapidly and responded with a much greater output of each of these constituents. As is the case with the injection of proteose the rate of injection is a factor of importance.

Dog 31 received a smaller dose than any of the others but the output of nitrogen and creatine was very much greater. This animal was very badly shocked. As is apparent from the experiments of Mendel, Underhill, and White<sup>2</sup> there is a considerable individual variation in response. The effect on Dogs 40 and 41 was characteristic.

The nitrogen output in all of these animals increased. It varied from 1 to 4 gm. in excess of the basal level, on the first day of the injection. The excretion continued above normal for several days thereafter (Chart 1). This is a clear argument for a significant tissue destruction.

The effect on the creatine output is a striking confirmation of the tissue destruction which the large nitrogen excretion indicates. It, moreover, points to the muscular tissue as the seat of this effect.

The evidence of the phosphates is not at once apparent, for the output above the amount injected does not seem large—no more than a few hundred mg. But the true condition is really obscured. Consideration of the protocols of the succeeding experiments on animal nucleic acids will show that the results are entirely negative as far as the effect on tissue destruction is concerned. We may consider those experiments from our view-

<sup>5</sup> Baumann, E. J., *J. Biol. Chem.*, 1918, xxxiii, p. xiv.

<sup>6</sup> Dr. Emil J. Baumann was kind enough to send us samples of spleen and yeast nucleic acids.

point, as controls. There, it will be observed, the phosphorus injected is not entirely eliminated in the urine. Not more than half the amount given is excreted. Hence, since in the experi-

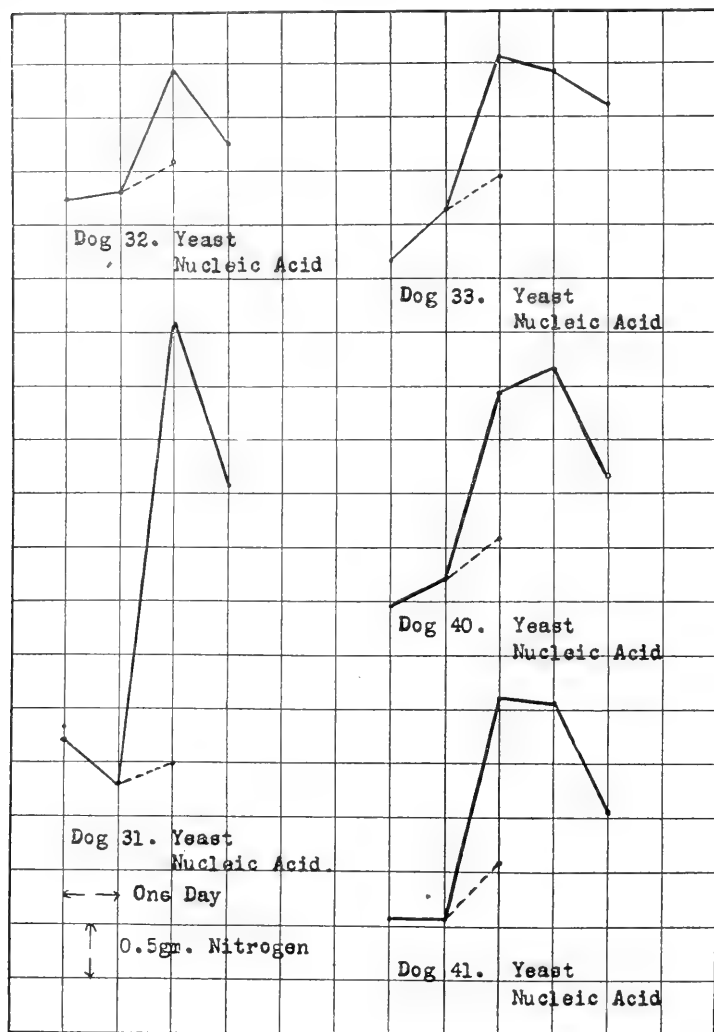


CHART 1. Influence of plant nucleic acid on the daily excretion of nitrogen. Dotted lines show amount of nitrogen injected.

TABLE I.  
*Influence of Plant Nucleic Acid.*

Dog.	Day.	Weight.	Urine vol- ume.	Total N.	Crea- tine.	P <sub>2</sub> O <sub>5</sub>	Remarks.
		<i>kg.</i>	<i>cc.</i>	<i>gm.</i>	<i>mg.</i>	<i>mg.</i>	
32	3		122	3.225	172	1,000	
	4	13.4	130	3.315	70	990	
	5		350	4.410	153	1,600	Injected slowly 125 cc. of yeast nucleic acid, 2 per cent solution (0.300 gm. N, 750 mg. of P <sub>2</sub> O <sub>5</sub> ).
	6		134	3.765	126	680	
	7						
33	3		104	3.180	52	887	
	4	13.04	92	3.645	42	962	
	5		340	5.040	240	2,000	Injected rapidly 116 cc. of yeast nucleic acid, 2 per cent solution (0.302 gm. N, 760 mg. of P <sub>2</sub> O <sub>5</sub> ).
	6		130	4.950	240	725	
	7		110	4.635	135	800	
31	4		92	2.235	132	413	
	5	11.00	96	1.825	194	507	
	6		200	6.050	237	1,250	Injected 50 cc. of 2 per cent yeast nucleic acid (0.120 gm. N, 320 mg. of P <sub>2</sub> O <sub>5</sub> ).
	7		188	4.550	462	500	
40	3		80	1.970	118	530	
	4	8.00	100	2.210	115	505	
	5		700	3.950	239	1,560	Injected 0.5 gm. per kilo of yeast nucleic acid (0.390 gm. N, 970 mg. of P <sub>2</sub> O <sub>5</sub> ).
	6		150	4.170	307	530	
	7		90	3.160			
41	3		125	3.590	138	613	
	4	12.7	100	3.590	65	760	
	5		685	5.150	200	1,790	Injected 0.5 gm. per kilo of yeast nucleic acid (0.540 gm. N, 1,350 mg. of P <sub>2</sub> O <sub>5</sub> ).
	6		210	5.100	380	810	
	7		160	4.040			

ments under discussion the output is not only as great as the amount injected but greater, we may conclude that there was a considerable excretion of phosphates above the basal level. This confirms the evidence derived from the consideration of the effect on the total nitrogen and the creatine.

It may be concluded, therefore, on grounds similar to those taken in the preceding paper that plant nucleic acid induces a marked tissue destruction when introduced directly into the circulation.

### *Effect of Animal Nucleic Acids.*

Contrary to our expectations we were unable to demonstrate a similar effect as a result of the injection of animal nucleic acids. In Table II and Chart 2 are recorded the results of these experiments.

In the five experiments where pure preparations from various sources were used, the results were uniformly negative with regard to the output of nitrogen. The amount injected was excreted, no more. Dog 37 showed a slight rise, this result, however, could not be repeated in Dog 38. The phosphate output to which we have already alluded, is in conformity with this finding. In Dogs 34 and 35 there were significant increases in the output of creatine. It is difficult to interpret these findings standing alone, unsupported by evidence from the nitrogen and phosphate output. We are inclined to disregard them, especially in view of the fact that this increase did not occur uniformly.

It may therefore be concluded that animal nucleic acid injected directly into the circulation unlike plant nucleic acid gives no evidence of inducing tissue destruction. In short, it is not a toxic substance.

### DISCUSSION.

This investigation, as was stated in the preceding paper, was undertaken primarily in order to find substances other than proteoses that are capable of stimulating catabolism and that might be formed as a result of tissue injury. We have demonstrated that plant nucleic acid is such a substance. It is reasonable to believe in view of the fact that the bacterial cell is largely composed of nucleoprotein, that the nucleic acids resulting from the

TABLE II.

*Influence of Animal Nucleic Acids.*

Dog.	Day.	Weight.	Urine volume.	Total N.	Crea- tine.	P <sub>2</sub> O <sub>5</sub>	Remarks.
		<i>kg.</i>	<i>cc.</i>	<i>gm.</i>	<i>mg.</i>	<i>mg.</i>	
34	4		110	1.800	175	463	
	5	10.5	110	1.945	120	463	
	6			2.190	235	960	Injected 0.3 gm. per kilo of thymus nucleic acid in 50 cc. (0.369 gm. N, 920 mg. of P <sub>2</sub> O <sub>5</sub> ).
	7		120	1.920	87	410	
35	4		105	3.240	20	600	
	5	11.4	110	3.150	42	600	
	6		90	2.955	100	835	Injected 50 cc. of thymus nucleic acid, 0.25 gm. per kilo (0.360 gm. N, 900 mg. of P <sub>2</sub> O <sub>5</sub> ).
	7		109	3.000	51	538	
36	4		97	3.340	43	613	
	5	11.5	116	3.250	10	535	
	6		190	3.602	79	755	Injected 0.1 gm. per kilo of dog nucleic acid (pan- creas and spleen, 0.158 gm. N, 400 mg. of P <sub>2</sub> O <sub>5</sub> ).
	7		170	3.450	109	535	
	8		115	2.860	30		
37	4		110	2.800	88	710	
	5	17.0	135	2.940	102	663	
	6		224	3.550	96	1,065	Injected 3 per cent solu- tion of spleen nucleic acid (0.320 gm. N, 800 mg. of P <sub>2</sub> O <sub>5</sub> ).
	7		137	3.420		325	
	8		115	2.820	76	362	
38	4		132	3.190	96	638	
	5	15.0	114	2.920	67	675	
	6		400	3.450	69	900	Injected 0.4 gm. per kilo of spleen nucleic acid (0.562 gm. N, 1,400 mg. of P <sub>2</sub> O <sub>5</sub> ).
	7		135	2.880	150	650	

decomposition of this substance might, in septic processes, be a contributing agent in the general intoxication. However, this source of nucleic acid is conceivably not great. The host's own nucleoproteins would be a much richer source, and as Whipple and Cooke and their coworkers have shown for proteoses, the destruction of the host's tissues is the real source of the intoxicat-

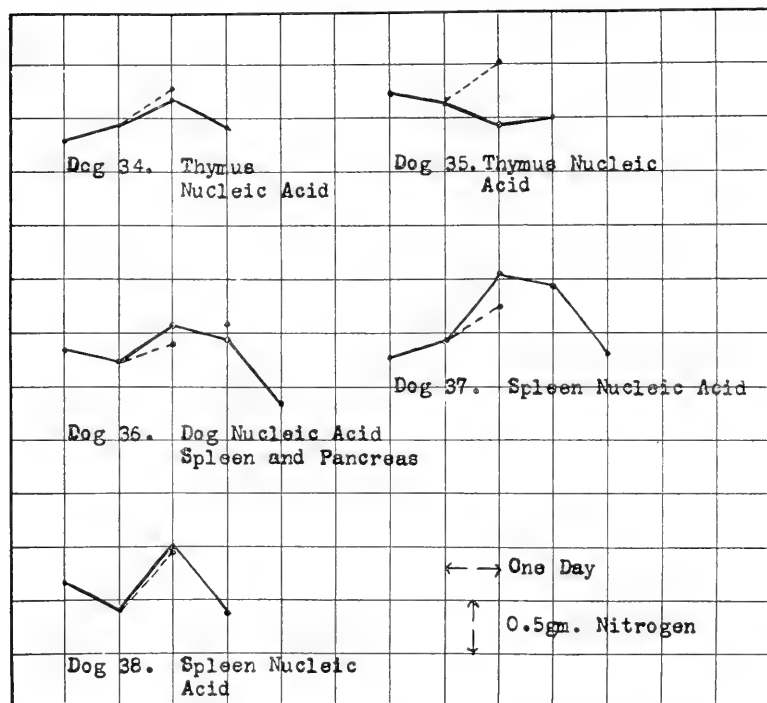


CHART 2. Influence of animal nucleic acids on the daily excretion of nitrogen. Dotted lines show the amount of nitrogen injected.

ing substance. Since we have demonstrated that animal nucleic acid is not toxic it is probable that this type of compound is not an important factor in the intoxication of inflammatory processes. Moreover, bacterial nucleic acid has been little studied and it is not clear whether it would behave like the animal or plant variety. Hence, little can be definitely argued for the rôle of nucleic acids, in inflammatory intoxications.

But our finding with regard to the failure of animal nucleic acid to injure tissue, opens the question of whether this compound resembles proteoses in physiological behavior at all. Mendel, Underhill, and White<sup>2</sup> showed that the plant nucleic acid they used, like proteose, caused a fall in pressure, delayed coagulability of the blood, increased flow of lymph, etc. We have recently

TABLE III.  
*Acute Effects of Plant and Animal Nucleic Acids.*

Dog.	Time.	Blood pressure.	Clotting time.	Hemoglobin.	Alkali reserve CO <sub>2</sub> volume.	Remarks.
	<i>min.</i>	<i>mm. Hg</i>		<i>per cent</i>	<i>per cent</i>	
30	0	130	3 min.	100	65	Injected spleen nucleic acid, 0.3 gm. per kilo in normal saline solution.
	1					
	2	60				
	3	130	3 hrs.	92	65	
	63	130		93		
	300	130	45 min.	100	59	
32	0	97	12 min.	100	58	Injected spleen nucleic acid, 0.5 gm. per kilo.
	1					
	2	30	3 hrs.	92	50	
	7	60				
	12	80				
	27	112		97	55	
	180	115	50 min.	101	53	
21	0	108	4 min.	100	41	Injected yeast nucleic acid, 0.3 gm. per kilo.
	1					
	2	10	11 min.	86		
	12	15		92	28	
	32	55	5 min.	96	37	
	92	22		115	20	
	152	10		128	15	

been able to add a concentrated blood<sup>7</sup> as measured by the increase in hemoglobin, and an acidosis<sup>7</sup> as measured by the alkali reserve.

On repeating this work with pure animal nucleic acid we found that the effect on the blood pressure was exceedingly fleeting and not very profound, that in some cases the coagulability was markedly delayed but that there was no effect on the blood concentra-

<sup>7</sup> Authors' unpublished data.



tion (hence probably none on the lymph flow) and no effect on the alkali reserve. Table III gives three typical protocols, two of animal nucleic acid and the other of plant nucleic acid for comparison. We used large doses and concentrated solutions of spleen nucleic acid neutralized before injection. Hence it must be concluded that animal nucleic acid differs also from plant nucleic acid in not showing the typical peptone type of shock. This finding is at variance with the work of Bang<sup>8</sup> who worked with guanylic acid of the pancreas.

#### SUMMARY.

1. Yeast nucleic acid induces a destruction of tissue, when introduced directly into the circulation, as evidenced by an increased output of nitrogen, creatine, and phosphates.

2. Animal nucleic acid has no such effect.

3. Animal nucleic acid differs also from plant nucleic acid in failing to give typical peptone type of shock.

4. Nucleic acids probably play a small rôle in the intoxication of inflammatory processes.

<sup>8</sup> Bang, I., quoted by Kossel, A., *Z. physiol. Chem.*, 1900-01, xxxi, 410; 1901, xxxii, 201.



## STUDIES ON THE PHYSIOLOGICAL ACTION OF SOME PROTEIN DERIVATIVES.

### IX. ALKALI RESERVE AND EXPERIMENTAL SHOCK.

By FRANK P. UNDERHILL AND MICHAEL RINGER.

*(From the Department of Pharmacology and Toxicology, Yale University, New Haven.)*

(Received for publication, August 1, 1921.)

The relation of acidosis to shock has been the subject of numerous investigations leading to a diversity of views. That a diminished alkali reserve may occur in shock no one will deny. On the other hand, evidence of a depleted alkali reserve in shock may be lacking. From a survey of all the evidence one is forced to the conclusion that the condition of acidosis in shock should be viewed from the aspect of an accompanying factor rather than as a contributing cause to the condition. It should be emphasized, however, that with shock once established an accompanying acidosis may directly contribute to a fatal outcome.

An investigation of shock induced by various protein derivatives has afforded us the opportunity of studying the relation of diminished alkali reserve to the shock-like condition evoked.

#### *Methods.*

The experiments were carried out upon full grown, well nourished dogs that were allowed to fast for a period of 24 hours previous to the experiment. Anesthesia was produced by a mixture of morphine sulfate (0.01 gm. per kilo), atropine sulfate (0.001 gm. per kilo), and ether. Blood pressure was recorded with a mercury manometer attached to the right carotid artery. Injections were made into a jugular vein. Unless otherwise specified all injections were made rapidly—within a minute—and the volume of fluid introduced did not exceed 50 cc. Blood

for analysis was drawn from a femoral artery and alkali reserve was determined by the method of Van Slyke. Witte peptone, purified proteoses, Vaughan's crude soluble poison, and nucleic acid were injected in doses of 0.3 to 0.5 gm. per kilo. Histamine dosage varied from 1 to 2 mg. per kilo calculated as the base.

*The Relation of Alkali Reserve to Shock Produced by Various Protein Derivatives.*

As may be observed from the table a shock-like condition as measured by low blood pressure was induced by the intravenous injection either of Witte's peptone, Vaughan's crude soluble poison, purified proteoses from casein, nucleic acid, or histamine hydrochloride. The data are arranged to emphasize the relation of the height of blood pressure and duration of low pressure to the alkali reserve. Thus, the word "maintained" in the table indicates a continued very low pressure, varying from 10 to 30 mm. of mercury. The word "temporary" designates a fall of pressure perhaps even to a very low point but with either a progressive or rapid rise to near normal limits. In general it may be noted that in these experiments, especially with Witte peptone, where low pressure has been maintained for a significant interval the alkali reserve shows the greatest decline. This, however, is by no means invariable. Generally, also, even though the fall in pressure is only temporary there is some indication of a decrease in alkali reserve. A striking difference is shown between the influence of yeast nucleic<sup>1</sup> acid and that of animal origin. In other respects the same fundamental difference in the behavior of these two types of nucleic acid when introduced into the body has been observed.<sup>2</sup> Less influence upon alkali reserve is to be seen from the injection of histamine than from that of any other substances introduced.

<sup>1</sup> Kindly furnished by Dr. E. J. Baumann.

<sup>2</sup> Ringer, M., and Underhill, F. P., *J. Biol. Chem.*, 1921, xlviii, 523.

*The Relation of Low Blood Pressure to Alkali Reserve.*

Experiment.	Low blood pressure.	Alkali reserve (volumes per cent).		Fate.
		Normal.	Later.	
Witte peptone.				
2	Maintained.	67	25	Died.
4	"	59	43	"
5	"	57	15	"
6	"	58	24	"
9	"	68	30	"
45	"	44	22	"
3	Temporary.	63	58	
17	"	46	37	
43	"	53	42	
47	"	51	33	
Deuteroproteoses (casein).				
33	Temporary.	54	34	
Vaughan's crude soluble poison (casein).				
19	Maintained.	50	27	
24	"	43	32	
36	"	50	46	
18	Temporary.	48	33	
35	"	61	34	
20	"	52	36	
38	"	59	43	
Nucleic acid (yeast).				
21	Maintained.	41	15	
Nucleic acid (thymus).				
32	Temporary.	58	53	
30	"	65	59	
29	"	55	54	
15	"	53	49	
28	"	50	35	
Histamine (hydrochloride).				
8	Maintained.	59	32	
11	"	58	42	
13	"	51	60	
14	"	50	50	

Why is it that there is such a diversity of response in the experiments presented? Undoubtedly in all the observations cited, with the possible exception of thymus nucleic acid, a shock-like condition intervened. This shock-like condition is accompanied by disturbances in respiration and circulation which may give rise to the production of acid products. Sufficient production of acid substances in the time interval possible under the experimental conditions hardly seems an adequate explanation. It seems to the authors that a much more plausible explanation lies in a decrease in the capacity of the body to excrete acid whether formed at a normal or at an accelerated rate. Thus a characteristic of low pressure is the appearance of a condition of anuria with a consequent greatly diminished ability to eliminate acid. We would suggest therefore that one large factor for the decrease in alkali reserve in the observations submitted is the failure of the renal mechanism incident to the low pressure. Inspection of the data from this view-point reveals a fairly close correlation between maintained low pressure and decreased alkali reserve. An apparent lack of correlation is encountered with the low pressure induced by histamine. An analysis of the details of the blood pressure fluctuations demonstrates that the pressure in the histamine experiments could not be maintained below 30 mm. of Hg even though histamine were continually introduced. In many of the experiments with other substances employed especially Witte peptone, a lower pressure obtained. Cushny<sup>3</sup> states that urine may continue to be secreted at 30 to 40 mm. of Hg. We would therefore suggest that the fall of alkali reserve noted in these experimental conditions of shock is directly related to failure of the renal mechanism to excrete acid—this inability of the kidney being induced by a maintained low blood pressure. Further work in this direction is in progress.

*Addendum.*—Since the above was written a paper has appeared by Eggstein (Eggstein, *J. Lab. and Clin. Med.*, 1921, vi, 481) on alkali reserve and protein shock. Our own experiments in as far as they are comparable yield results in many respects in confirmation of those of Eggstein.

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<sup>3</sup> Cushny, A. R., *The secretion of the urine*, New York and London, 1917.

## STUDIES ON THE PHYSIOLOGICAL ACTION OF SOME PROTEIN DERIVATIVES.

### X. THE INFLUENCE OF NUCLEIC ACID ON THE METABOLISM OF THE FASTING RABBIT.\*

BY FRANK P. UNDERHILL AND MARY LOUISA LONG.

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New Haven.)

(Received for publication, August 1, 1921.)

In a previous paper<sup>1</sup> in this series it has been pointed out that the introduction of yeast nucleic acid into the circulation of the fasting dog induces a destruction of tissue as evidenced by an increased output of nitrogen, creatine, and phosphates. The acute effects of this nucleic acid resemble strongly those characteristic of peptone shock. Since rabbits are more or less refractory to peptone injection in this respect experiments similar to those for dogs previously reported have been carried through on rabbits with nucleic acid to determine whether the nucleic acid influence as seen in dogs is capable of being extended to the rabbit.

#### *Methods.*

The animals were fasted for 2 days previous to the injection. Urinary excretion was divided into 24 hour periods by pressure upon the bladder through the abdominal wall. Total nitrogen determinations were made in duplicate by the Kjeldahl method, preformed creatinine by Folin's method, and total creatinine by the procedure of Benedict. Phosphates were estimated by titration with uranium acetate. Blood drawn from an ear vein was precipitated and analyzed for non-protein nitrogen by the method

\* The data of this paper are taken from the essay by Mary Louisa Long in partial fulfillment of the requirements for the degree of Master of Science, Yale University, 1920.

<sup>1</sup> Ringer, M., and Underhill, F. P., *J. Biol. Chem.*, 1921, *xlvi*, 523.

of Folin and Wu. Hemoglobin was determined by the procedure of Cohen and Smith. Nucleic acid, containing 12.2 per cent nitrogen and 8.2 per cent phosphorus was prepared from brewers' yeast by the method of Baumann.<sup>2</sup> A fresh 1 per cent solution was used for each injection made by dissolving the nucleic acid in hot 0.9 per cent NaCl solution with the aid of a few drops of concentrated KOH. The injections were made on the basis of nitrogen content of the solution. Amounts varying from 20 to 60 cc. according to the size of the animal were slowly injected into the circulation through the marginal ear vein. The immediate effects of the injection were an accelerated respiration and a transitory shock-like condition.

### *Control Experiments.*

#### *The Influence of Fasting, Etc., upon Nitrogen Excretion in Rabbits.*

To determine whether the increased nitrogen output caused by the injection of nucleic acid really means an increased tissue catabolism, or is due to other factors, such as fasting, rabbits in a fasting condition were injected with a slightly alkaline solution. The following experiments show that under these conditions the nitrogen rises slightly on the 2nd day of fasting, but afterwards keeps on a level or falls. The creatinine remains practically unchanged, but there is a slight rise in creatine. The phosphates show practically no change.

The nitrogen gradually increases, showing a marked rise on the last 2 days of the experiment. This probably accounts for a secondary rise observed in some of the injected rabbits. The creatinine keeps on a level as in the saline injected animals, but the creatine rises very markedly, increasing to eight times its original amount on the last day of fasting. This no doubt indicates a destruction of muscular tissue, and was usually observed in very thin animals. The phosphates remained on a level as before.

<sup>2</sup> Baumann, E. J., *Proc. Soc. Exp. Biol. and Med.*, 1919-20, xvii, 118.



*The Influence of Intravenous Administration of Nucleic Acid upon Nitrogen Excretion in the Rabbit.*

In testing the influence of the intravenous injection of nucleic acid upon nitrogen excretion, varying amounts were given in order to determine the most effective dose. Rabbits 8 and 9

TABLE I.

*The Influence of Fasting, Etc., upon Nitrogen Output. Control Experiments.*

Date.	Water intake.	Urine.					Remarks.
		Volume.	Total nitrogen.	Creatinine.	Creatine.	P <sub>2</sub> O <sub>5</sub>	
Rabbit 6. Body weight 1.8 kilos.							
1920	cc.	cc.	gm.	gm.	gm.	gm.	
Mar. 8	200	55	0.584	0.050	0.004	0.150	Mar. 9, injection of 67 cc. of faintly alkaline 0.9 per cent NaCl solution.
" 9	90	90	0.714	0.063	0.011	0.204	
" 10	37	105	0.602	0.044	0.010	0.150	
" 11	80	72	0.882	0.065	0.030	0.336	
" 12	80	52	0.884	0.058	0.043	0.226	
Rabbit 22. Body weight 1.85 kilos.							
Apr. 29	40	70	0.562	0.069	0.038	0.202	Apr. 30, injection of 52 cc. of faintly alkaline 0.9 per cent NaCl solution.
" 30	55	60	0.942	0.072	0.018	0.192	
May 1	0	50	0.850	0.099	0.015	0.258	
" 2	0	34	0.872	0.084	0.006	0.202	
" 3	40	23	0.854	0.079	0.011	0.182	
Rabbit 23. Body weight 1.62 kilos.							
May 2	170	75	1.110	0.108	0.013	0.162	May 3, injection of 40 cc. of faintly alkaline 0.9 per cent NaCl solution.
" 3	100	98	0.874	0.069	0.008	0.180	
" 4	80	100	1.035	0.075	?	0.293	
" 5	0	32	0.756	0.065	0.036	0.222	
" 6	0	30	0.765	0.073	0.026	0.190	

were given very small doses of nucleic acid, 0.05 gm. per kilo of body weight with practically no effect, as shown in Table III.

The picture is the same as shown in the control experiments. The fact that the nitrogen rises to a maximum the day after the injection and then returns to normal might indicate a slight destructive action of a small dose.

Table IV shows the effect of increasing the amount of nucleic acid injected. Rabbit 7 received 0.1 gm. per kilo of body weight. The nitrogen rose 0.4 gm., an amount not to be accounted for by

TABLE II.  
*Effect of Plain Fasting on Nitrogen Output.*

Date.	Water intake.	Urine.					Remarks.
		Vol- ume.	Total nitro- gen.	Creati- nine.	Crea- tine.	P <sub>2</sub> O <sub>5</sub>	
Rabbit 19. Body weight 1.68 kilos.							
<i>1920</i>	<i>cc.</i>	<i>cc.</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	
Apr. 17	0	32	0.474	0.054	0.008	0.356	
“ 18	100	30	0.589	0.046	0.008	0.298	
“ 19	5	32	0.726	0.052	0.015	0.230	
“ 20	85	55	1.056	0.058	0.051	0.258	
“ 21	100	130	1.697	0.056	0.074	0.363	

TABLE III.  
*Effect of Injection of 0.05 Gm. of Nucleic Acid per Kilo.*

Date.	Water intake.	Urine.					Remarks.
		Vol- ume.	Total nitro- gen.	Creati- nine.	Crea- tine.	P <sub>2</sub> O <sub>5</sub>	
Rabbit 8. Body weight 2.7 kilos.							
1920	cc.	cc.	gm.	gm.	gm.	gm.	
Mar. 14	0	56	0.938	0.095	0.013	0.186	Mar. 14, injection of a
" 15	80	86	0.977	0.095	0.009	0.247	1 per cent nucleic acid
" 16	0	150	1.045	0.096	0.014	0.278	solution = 0.0216 gm.
" 17	100	82	0.951	0.098	0.020	0.296	of nitrogen.
Rabbit 9. Body weight 2.4 kilos.							
Mar. 25	0	60	0.876	0.062	0.005	0.356	Mar. 26, injection of 15
" 26	60	50	0.969	0.070	0.038	0.284	cc. of a 1 per cent nu-
" 27	60	36	1.072	0.101	0.029	0.220	cleic acid solution =
" 28	100	35	0.933	?	?	0.304	0.0192 gm. of nitrogen.
" 29	70	35	0.920	?	?	0.184	

the small amount of nitrogen injected in the nucleic acid solution. The creatinine, creatine, and phosphates were unaffected by the injection.

In Table V a very marked effect on the urinary nitrogen is shown for Rabbits 3 and 14, after an injection of 0.2 gm. of

TABLE IV.

*Effect of Injection of 0.1 Gm. of Nucleic Acid on Nitrogen Output.*

Date.	Water intake.	Urine.					Remarks.
		Vol- ume.	Total nitro- gen.	Creati- nine.	Crea- tine.	P <sub>2</sub> O <sub>5</sub>	
Rabbit 7. Body weight 1.8 kilos.							
1920	cc.	cc.	gm.	gm.	gm.	gm.	
Mar. 13	0	48	0.462	0.070	0.015	0.104	Mar. 14, injection of
" 14	35	44	0.690	0.071	0.010	0.172	22.3 cc. of a 1 per cent
" 15	40	120	1.066	0.081	0.024	0.207	nucleic acid solution =
" 16	0	40	0.774	0.053	0.009	0.167	0.016 gm. of nitrogen.
" 17	100	76	0.558	0.063	0.028	0.251	

TABLE V.

*Effect of Injection of 0.2 Gm. of Nucleic Acid per Kilo.*

Date.	Water intake.	Urine.					Remarks.
		Volume.	Total nitrogen.	Creatinine.	Creatinine.	P <sub>2</sub> O <sub>5</sub>	
Rabbit 3. Body weight 1.9 kilos.							
1920	cc.	cc.	gm.	gm.	gm.	gm.	
Feb. 12	200	13	0.354	0.043	0.005	0.045	Feb. 13, injection of 38
" 13	55	46	0.427	0.084	0.005	0.102	cc. of a 1 per cent nu-
" 14	0	126	1.431	0.093	0.099	0.105	cleic acid solution =
" 15	100	140	2.196	0.090	0.166	0.390	0.049 gm. of nitrogen.
" 16	25	90	1.425	0.032	0.155	0.288	
Rabbit 14. Body weight 1.6 kilos.							
Apr. 5	70	32	0.708	0.060	0.019	0.130	Apr. 6, injection of 40
" 6	100	60	0.788	0.049	0.011	0.178	cc. of a 1 per cent nu-
" 7	20	134	1.120	0.034	0.088	0.258	cleic acid solution =
" 8	100	77	1.359	0.046	0.052	0.318	0.051 gm. of nitrogen.
" 9	100	100	1.947	0.042	0.105	0.386	

nucleic acid per kilo of body weight. In Rabbit 3 the nitrogen is increased 1 gm. on the day after injection, and rises still higher on the next day. The creatinine is not changed markedly, but

the creatine goes up tremendously, indicating a great tissue destruction. It is interesting to note that the rise in phosphates comes on the 3rd day after injection. Rabbit 14 did not show as striking effects as Rabbit 3. The nitrogen increased 0.5 gm. on the day following injection and continued to rise on the following days. Creatine is quadrupled, and the phosphates are steadily increased; however, this and the high increase in nitrogen on the last 2 days are probably fasting factors.

TABLE VI.

*Effect on Urinary Nitrogen after Injection of 0.3 Gm. of Nucleic Acid per Kilo.*

Date.	Water intake.	Urine.					Remarks.
		Vol- ume.	Total nitro- gen.	Creati- nine.	Crea- tine.	P <sub>2</sub> O <sub>5</sub>	
Rabbit 5. Body weight 1.76 kilos.							
1920	cc.	cc.	gm.	gm.	gm.	gm.	
Mar. 8	200	53	1.184	0.063	0.028	0.140	Mar. 9, injection of 65.5
" 9	105	105	1.475	0.082	0.063	0.283	cc. of a 1 per cent nu-
" 10	42	168	1.876	0.096	0.086	0.406	cleic acid solution =
" 11	100	115	1.877	0.053	0.101	0.442	0.084 gm. of nitrogen.
" 12	100	150	1.246	0.071	0.197	0.487	
Rabbit 4. Body weight 2.4 kilos.							
Feb. 16	15	59	0.636	0.095	0.031	0.096	Feb. 17, injection of 72
" 17	0	38	0.894	0.102	0.021	0.161	cc. of a 1 per cent solu-
" 18	30	105	1.189	0.126	0.078	0.160	tion of nucleic acid =
" 19	10	56	0.650	0.108	0.000	0.232	0.086 gm. of nitrogen.
" 20	35	31	0.621	0.092	0.003	0.206	

Rabbits 4 and 5 were given 0.3 gm. of nucleic acid per kilo. There is a decided rise in both the nitrogen and creatine, as shown in Table VI, but the increase is not as marked as with the 0.2 gm. dose, indicating that larger amounts are no more potent.

These experiments show that the intravenous injection of nucleic acid produces a marked rise in the urinary nitrogen and creatine, and that amounts of 0.2 gm. per kilo of body weight give the most striking results.

*The Influence of Intravenous Injection of Nucleic Acid upon the Non-Protein Nitrogen of the Blood.*

Since the results on urinary nitrogen determinations point toward an increased protein catabolism it is of interest to follow the non-protein nitrogen of the blood, to determine whether there is an accumulation of the catabolic products in the blood. To interpret the results correctly, the hemoglobin curve was likewise followed to note whether there was any blood volume change.

TABLE VII.

*Influence of Intravenous Injection of Nucleic Acid on the Hemoglobin of the Blood.*

Date.	Hemoglobin of the blood.		Remarks.
	Time of drawing blood.	Hemoglobin.	
Rabbit 14. Body weight 1.6 kilos.			
1920		per cent	
Apr. 6	Before injection.	81	Animal fasted 2 days previous to an injection of 40 cc. of a 1 per cent solution of nucleic acid = 0.051 gm. of nitrogen.
	2 hrs. after "	70	
	4 " " "	57	
	6 " " "	55	
	8 " " "	53	
" 7	24 " " "	61	
	27 " " "	61	
" 8	48 " " "	65	

An increase in volume, represented by a low hemoglobin would mean a decrease in concentration, and if the non-protein nitrogen did not likewise fall in amount, a real increase of the latter might be indicated. There was some question as to whether the frequent bleeding might not have an effect on the volume; consequently hemoglobin determinations only were made on Rabbit 14 after an injection of nucleic acid. Table VII shows that the volume decrease of about 20 per cent is a result of the injection, and not of the bleeding.

The blood of Rabbit 15 was analyzed for non-protein nitrogen. The results, as given in Table VIII, show an increase of about 5 per cent in the first 3 hours, and in view of the fact that the injection causes a dilution of the blood, the increase is still greater.

Table IX gives a complete picture of the urinary and blood nitrogen after an injection of 0.2 gm. of nucleic acid per kilo in two different rabbits. The urine picture is practically the same as seen in the rabbits previously discussed. The increase in nitrogen on the day following the injection coincides with the accumulation of non-protein nitrogen in the blood within the first 12 hours after injection. In Rabbit 16 the non-protein nitrogen remains practically on the level, but the corresponding decrease in hemoglobin would indicate that there is a marked rise. Rabbit 17 shows a slight increase in non-protein nitrogen, but a decided decrease in hemoglobin, showing a very marked

TABLE VIII.

*Influence of Intravenous Injection of Nucleic Acid on the Non-Protein Nitrogen of the Blood.*

Date.	Non-protein nitrogen.		Remarks.
	Time of drawing blood.	Per 100 cc.	
Rabbit 15. Body weight 1.5 kilos.			
1920		mg.	
Apr. 6	Before injection.	55	Animal fasted 2 days previous to an injection on Apr. 6 of 37 cc. of a 1 per cent solution of nucleic acid = 0.048 gm. of nitrogen.
	3 hrs. after "	61	
	6 " " "	59	
	9 " " "	48	
" 7	24 " " "	56	
	27 " " "	56	
" 8	48 " " "	57	

rise. The marked increase on the last 2 days of the experiment may represent a fasting factor. The animal was very thin and apparently undernourished.

These experiments show that the increase of urinary nitrogen is accompanied by an accumulation of protein catabolic products in the blood.

*The Influence of Repetition of Nucleic Acid Injections upon Nitrogen Excretion in Rabbits.*

Rabbit 11 received the same dose of nucleic acid as was injected a week earlier. The increase in nitrogen output is just as marked or more so, as after the first injection, although the rise comes on

TABLE IX.  
*Influence of Intravenous Injections of Nucleic Acid on the Urinary and Blood Nitrogen.*

Date.	Urine.				Blood.		Remarks.		
	Water intake.	Vol-ume.	Total nitro-gen.	Creati-nine.	Crea-tine.	P <sub>2</sub> O <sub>5</sub>		Time of drawing blood.	Hemo-globin.
1920	cc.	cc.	gm.	gm.	gm.	gm.		per cent	mg.
Apr. 9	30	32	0.700	0.072	0.053	0.250	Before injection.	117.6	38.59
							1½ hrs. after "	100.0	
							3 "	" "	95.2
							6 "	" "	42.12
							9 "	" "	97.6
							12 "	" "	34.24
							24 "	" "	88.3
" 10	0	83	?	0.090	0.078	0.152	" "	" "	41.04
							30 "	" "	82.2
								" "	46.36
								" "	85.7
								" "	32.00
								" "	86.6
								" "	39.40
Rabbit 16. Body weight 2.3 kilos.									
Animal fasted 2 days previous to an injection on Apr. 9 of 58.3 cc. of a 1 per cent nucleic acid solution = 0.075 gm. of nitrogen.									
Apr. 9	100	84	0.252	0.046	0.004	0.146	Before injection.	130.4	37.20
" 10	0	38	0.540	0.048	0.002	0.184	3 hrs. after "	100.0	54.80
							6 "	" "	?
							9 "	" "	82.2
							24 "	" "	58.80
" 11	0	104	0.949	0.048	0.057	0.195	30 "	" "	69.8
								" "	68.36
								" "	83.32
Rabbit 17. Body weight 1.4 kilos.									
Apr. 10, injection of 35 cc. of a 1 per cent nucleic acid solution = 0.045 gm. of nitrogen.									
Apr. 9	100	84	0.252	0.046	0.004	0.146	Before injection.	130.4	37.20
" 10	0	38	0.540	0.048	0.002	0.184	3 hrs. after "	100.0	54.80
							6 "	" "	?
							9 "	" "	82.2
							24 "	" "	58.80
" 11	0	104	0.949	0.048	0.057	0.195	30 "	" "	69.8
								" "	68.36
								" "	83.32

the 2nd day after injection. The phosphates are likewise increased, but the amount of creatine remains practically unchanged, leading one to believe that perhaps immunity is established, as the indications are that the tissue catabolism is not so great. Rabbit 10 shows the result of repeating the dose a month later. The effect is quite similar to that shown for Rabbit 11. The nitrogen output is doubled on the 2nd day after injection, but no marked increase is shown in the creatine.

TABLE X.  
*Repetition of Nucleic Acid Injection.*

Date.	Water intake.	Urine.					Remarks.
		Vol- ume.	Total nitro- gen.	Creati- nine.	Crea- tine.	P <sub>2</sub> O <sub>5</sub>	
Rabbit 11. Body weight 1.8 kilos.							
1920	cc.	cc.	gm.	gm.	gm.	gm.	
Mar. 25	0	60	0.529	0.056	0.006	0.194	Immunity experiment on Rabbit 7, 1 week later. Mar. 26, injection of 22.3 cc. of a 1 per cent nucleic acid solution = 0.029 gm. of nitrogen.
" 26	10	35	0.603	0.059	0.041	0.232	
" 27	0	52	0.768	0.063	0.012	0.103	
" 28	20	110	1.245	0.058	0.046	0.410	
" 29	0	45	1.578	0.039	0.042	0.308	
Rabbit 10. Body weight 2.24 kilos.							
Mar. 17	0	90	1.080	0.057	0.017	0.090	Immunity experiment on Rabbit 4, 1 month later. Mar. 18, injection of 83.3 cc. of a 1 per cent nucleic acid solution = 0.108 gm. of nitrogen.
" 18	100	100	?	0.066	0.001	0.165	
" 19	0	125	0.945	0.081	0.009	0.258	
" 20	0	135	1.701	0.081	0.024	0.324	
" 21	100	110	2.300	0.073	0.062	0.187	

#### SUMMARY.

The experiments reported point toward a marked tissue catabolism, chiefly shown in the urinary nitrogen and creatine. There is likewise a rise in the non-protein nitrogen of the blood, which is not so marked. The fact that non-protein nitrogen is being introduced with the nucleic acid injection must be taken into consideration. This may amount to as much as is already



present in the blood as, for example, is the case with Rabbit 15. According to McQuarrie's figures on blood volume, a rabbit of 1.5 kilos would have about 100 cc. (6.5 cc. per 100 gm. of body weight), so that the introduction of 48 mg. of nitrogen into the blood stream would double the amount present, which was 55 mg. per 100 cc. of blood. But with normal kidneys, this should be eliminated quickly. The fact that the non-protein nitrogen does not rise very markedly may be explained by the excretion of the catabolic products about as rapidly as they are formed, so that there would be no great accumulation of these products at any one time. We would suppose this to be the case if the kidneys were working efficiently. The dilution of the blood following nucleic acid injection in the rabbit differs from the results obtained by Ringer and Underhill for the dog. In the dog a marked concentration may be present. It would appear in the rabbit experiments that the relatively large volume of fluid introduced is only slowly compensated for in this animal. In the dog compensation occurs relatively quickly.

#### CONCLUSION.

Injection of nucleic acid into the circulation of the fasting rabbit induces increased tissue catabolism, as indicated by the augmented output of urinary nitrogen and creatine.

In this respect, then, the dog and rabbit respond alike to the intravenous introduction of nucleic acid.

In the rabbit nucleic acid injection produces dilution of the blood, in the dog concentration.

In the rabbit there may be an increased non-protein nitrogen of the blood after nucleic acid injection.



## STUDIES ON THE PHYSIOLOGICAL ACTION OF SOME PROTEIN DERIVATIVES.

### XI. THE INFLUENCE OF SOME PROTEIN SPLIT PRODUCTS UPON THE METABOLISM OF FASTING RABBITS.\*

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(Received for publication, August 1, 1921.)

A previous communication<sup>1</sup> relative to the influence of various protein split products upon the metabolism of fasting dogs has shown that in general the higher members of hydrolytic change of protein induce increased catabolism as evidenced by a large output of urinary nitrogen, creatine, and phosphates. This is especially true for such substances as Witte peptone, purified proteoses, and Vaughan's crude soluble poison. In general, however, the dog is peculiarly susceptible to the influence of proteoses and proteose-like substances, and the character of response elicited by the introduction of such substances is sufficiently important in its bearing upon the problems of inflammatory processes to warrant the extension of this type of experiment to a species of animal recognized to be refractory to proteose injection. Such an animal is the rabbit.

#### *Methods.*

In general the methods followed were those outlined in a former paper<sup>2</sup> hence repetition is unnecessary.

\* The data are taken from theses presented by Philip Greenberg and Anthony F. Alu in partial fulfillment of the requirements for the degree of Doctor of Medicine, Yale University, 1920.

<sup>1</sup> Ringer, M., and Underhill, F. P., *J. Biol. Chem.*, 1921, xlviii, 503.

<sup>2</sup> Underhill, F. P., and Long, M. L., *J. Biol. Chem.*, 1921, xlviii, 537.

*The Influence of Intravenous Injections of Witte Peptone, and Proteoses upon Nitrogen Excretion.*

An extensive experience with the influence of fasting upon urinary excretion in the rabbit has demonstrated that this con-

TABLE I.

*The Influence of Witte Peptone on Urinary Nitrogen Output in a Rabbit of 2.0 Kilos Body Weight.*

Day.	Intake.	Volume.	Specific gravity.	Reaction.	Nitrogen.	Creatinine.	Creatine.	Phosphates.	Remarks.
	cc.	cc.			gm.	mg.	mg.	gm.	
1	130	15		Acid.	0.612			0.070	
2	0	27		"	0.662	83	9	0.073	
3	0	35		"	1.068	106	25	0.138	Injection of 0.4 gm. of Witte peptone per kilo (0.158 gm. of nitrogen). Volume 50 cc.
4	0	20		"	0.885	87	8	0.170	
5	110	58	1,030	"	0.969	85	6	0.169	

TABLE II.

*The Influence of Witte Peptone on Urinary Nitrogen Output in a Rabbit of 1.8 Kilos Body Weight.*

Day.	Intake.	Volume.	Specific gravity.	Reaction.	Nitrogen.	Creatinine.	Creatine.	Phosphates.	Remarks.
	cc.	cc.			gm.	mg.	mg.	gm.	
1	0	62	1,022	Acid.	0.704	815	92	0.070	
2	20	65	1,020	"	0.978	922	66	0.171	
3	25	95	1,021	"	1.038	125	51	0.152	Injection of 0.15 gm. of Witte peptone per kilo (0.05 gm. of nitrogen). Volume 50 cc.
4	25	83	1,024	"	1.716	164	71	0.287	
5	10	80	1,021	"	1.603	149	88	0.265	

dition contributes no complicating factor in the interpretation of results obtained after introduction of various solutions.<sup>2,3</sup> Repetition of such data, therefore, will be omitted.

<sup>3</sup> Also from many unpublished data.

Relative to the toxicity for rabbits of Witte peptone and purified proteoses<sup>4</sup> it may be stated that in no instances was there indication that the animals were even mildly ill. However, several animals died without definite symptoms.

TABLE III.

*The Influence of Witte Peptone on Urinary Nitrogen Output in a Rabbit of 2.1 Kilos Body Weight.*

Day.	Intake.	Volume.	Specific gravity.	Reaction.	Nitrogen.	Creatinine.	Creatine.	Phosphates.	Remarks.
	cc.	cc.			gm.	mg.	mg.	gm.	
1	40	57	1,040	Acid.	0.663	100	21	0.140	
2	90	96	1,021	"	0.675	98	8	0.199	
3	0	295	1,020	"	1.089	114	9	0.215	Injection of 0.4 gm. of Witte peptone per kilo (0.160 gm. of nitrogen). Volume 100 cc.
4	65	64	1,030	"	0.813	98	7	0.236	
5									

TABLE IV.

*The Influence of Deuterocaseose on Urinary Nitrogen Output in a Rabbit of 3.1 Kilos Body Weight.*

Day.	Intake.	Volume.	Specific gravity.	Reaction.	Nitrogen.	Creatinine.	Creatine.	Phosphates.	Remarks.
	cc.	cc.			gm.	mg.	mg.	gm.	
1			Specimen lost.						
2	0	47		Acid.	0.852	131	8	0.263	
3	10	65		"	1.233	181	47	0.314	Injection of 0.15 gm. of deuterocaseose per kilo (0.074 gm. of nitrogen). Volume 50 cc.
4	0	62	1.031	"	0.978	123	34	0.226	
5	100	46		"	0.834	104	28	0.194	

From the view-point of the influence of Witte peptone and purified proteoses upon nitrogenous metabolism the data given in Tables I to IV show quite clearly that there is an increase in

<sup>4</sup> The preparation of this substance has already been described. Cf. Underhill, F. P., and Ringer, M., *J. Pharmacol. and Exp. Therap.*, 1921, in press.

the output of urinary nitrogen. In most cases also there is a definite but smaller augmented excretion of creatine and phosphates. In spite then of the fact that the rabbit and dog show different responses to the acute effects of the introduction into the circulation of Witte peptone and purified proteoses these substances exert with both types of animal the same effect upon nitrogenous metabolism, a fact which would lead one to the view that the influence upon nitrogenous metabolism is not necessarily closely associated with the influence which produces the toxic symptoms.

*The Influence of Vaughan's Crude Soluble Poison and Vaughan's Non-Toxic Body upon Nitrogenous Metabolism.*

In Tables V and VI may be found illustrative data selected from many experiments obtained after injection of Vaughan's crude soluble poison prepared from egg white. An inspection of these tables will make it evident that the intravenous injection of Vaughan's crude soluble poison (neutralized) calls forth a significant increase in the urinary nitrogen output of the fasting rabbit. The augmented nitrogen excretion is accompanied by a corresponding increase in the phosphate elimination. Creatine and creatinine are not markedly influenced.

It will be recalled that in the preparation of Vaughan's crude soluble poison a portion of the protein employed as the source becomes insoluble in the alkaline alcoholic solution and does not possess toxic properties. Such a product obtained from casein when injected into guinea pigs was harmless. On the other hand, the intravenous introduction of this non-toxic substance into the rabbit induces just as great an increase in urinary nitrogen and phosphorus as comparable doses of Vaughan's crude soluble poison (see Tables VII and VIII). In other words the non-toxic material is just as efficacious in accelerating protein catabolism as is the poisonous portion. It must therefore be quite apparent that this influence upon protein catabolism is not specific for Vaughan's crude soluble poison nor is its influence necessarily associated with its toxic properties as evidenced by other symptoms.

TABLE V.

*The Influence of Vaughan's Crude Soluble Poison upon the Urinary Nitrogen Output in a Rabbit of 2.1 Kilos Body Weight.*

Day of fasting.	Water intake.	Urine.						Remarks.
		Volume.	Reaction to litmus.	Total nitrogen.	Phosphates $P_2O_5$ .	Creatinine.	Creatine.	
	cc.	cc.		gm.	gm.	mg.	mg.	
1	95	17	Acid.	0.756	0.024	85	11	
2	0	38	"	0.774	0.049	111	11	
3	0	35	"	0.874	0.064	88	17	Injection of 105 mg. of Preparation I ( = 0.10 gm. of nitrogen) dissolved in 100 cc. of 0.9 per cent NaCl = 50 mg. per kilo of body weight. Time, 10 minutes.
4	0	69	"	1.552	0.124	158	48	
5	0	50	"	1.050	0.120	160	50	

TABLE VI.

*The Influence of Vaughan's Crude Soluble Poison upon the Urinary Nitrogen Output in a Rabbit of 1.8 Kilos Body Weight.*

Day of fasting.	Water intake.	Urine.						Remarks.
		Volume.	Reaction to litmus.	Total nitrogen.	Phosphates $P_2O_5$ .	Creatinine.	Creatine.	
	cc.	cc.		gm.	gm.	mg.	mg.	
1	42	52	Acid.	0.732	0.215	82	4	
2	30	68	"	0.762	0.179	65	10	
3	5	114	"	1.018	0.250	62	35	Injection of 135 mg. of Preparation I ( = 0.14 gm. of nitrogen) dissolved in 0.9 per cent NaCl = 75 mg. per kilo of body weight. Time, 10 minutes.
4	0	28	"	0.693	0.163	65	69	
5	0	50	"	0.640	0.160	63	58	

TABLE VII.

*The Influence of Vaughan's Non-Toxic Protein upon the Urinary Nitrogen Output in a Rabbit of 2.1 Kilos Body Weight.*

Day of fasting.	Water intake.	Urine.						Remarks.
		Volume.	Reaction to litmus.	Total nitrogen.	Phosphates $P_2O_5$ .	Creatinine.	Creatine.	
	cc.	cc.		gm.	gm.	mg.	mg.	
1	0	54	Acid.	0.600	0.100	90	8	
2	0	42	"	0.780	0.184	87	15	
3	0	122	"	1.200	0.600	99	53	Injection of 210 mg. of non-toxic protein ( = 0.23 gm. of nitrogen) dissolved in 0.9 per cent NaCl = 100 mg. per kilo of body weight. Time, 10 minutes.
4	0	78	"	1.398	0.425	93	74	
5	0	80	"	2.432	0.425	88	92	

TABLE VIII.

*The Influence of Vaughan's Non-Toxic Protein upon the Urinary Nitrogen Output in a Rabbit of 2.3 Kilos Body Weight.*

Day of fasting.	Water intake.	Urine.						Remarks.
		Volume.	Reaction to litmus.	Total nitrogen.	Phosphates $P_2O_5$ .	Creatinine.	Creatine.	
	cc.	cc.		gm.	gm.	mg.	mg.	
1	40	62	Acid.	0.810	0.210	69	21	
2	0	45	"	0.880	0.165	64	33	
3	0	47	"	1.014	0.265	88	42	Injection of 115 mg. of non-toxic protein ( = 0.12 gm. of nitrogen) dissolved in 50 cc. of 0.9 per cent NaCl = 50 mg. per kilo of body weight. Time, 10 minutes.
4	0	45	"	0.942	0.238	72	55	
5	0	46	"	1.200	0.240	70	70	



## CONCLUSIONS.

In spite of the fact that the rabbit is refractory to the acute effects of Witte peptone and proteoses, the intravenous injection of these substances into the fasting animal induces an accelerating influence upon protein catabolism similar to the response obtained in dogs.

A similar influence is exerted by the intravenous injection of Vaughan's crude soluble poison and Vaughan's non-toxic body.

These facts lead to the view that the action exerted upon nitrogenous metabolism is not necessarily related to toxic properties possessed by some of these substances.

The influence seen upon nitrogenous metabolism is therefore not specific for a given protein derivative but is probably an indication of the detrimental action incident to the introduction into the circulation of a foreign protein.



## THE INFLUENCE OF THYROPARATHYROIDECTOMY UPON BLOOD SUGAR CONTENT AND ALKALI RESERVE.

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(Received for publication, August 1, 1921.)

In a recent communication Hastings and Murray<sup>1</sup> report their failure to corroborate the work of Underhill and Blatherwick<sup>2,3</sup> who showed that blood sugar content is low after thyroparathyroidectomy. They state that "their (Underhill and Blatherwick) determinations made with the method of Forschbach and Severin showed extraordinary variations and were not accompanied by sufficient data to allow for much comment." In reply it may be stated that the only data essential for the understanding of the problem were the changes in blood sugar content and their correlation to the occurrence of tetany and these were given in full. No mention is made of the experiments of Underhill and Blatherwick<sup>2</sup> wherein blood sugar estimations were made by the method of Vosburgh and Richards<sup>4</sup> which involves the actual weighing of cuprous oxide. It is quite fair to admit that results obtained by the Forschbach and Severin method may be subject to criticism because of the small quantity of blood employed in this colorimetric procedure. No such criticism can, however, be applied to the method of Vosburgh and Richards especially since quantities of blood varying from 20 to 60 gm. were employed for sugar estimations.

Hastings and Murray employed the MacLean method of sugar estimation and assert that "in our series, at least, there was no marked disturbance in sugar metabolism for the first few days

<sup>1</sup> Hastings, A. B., and Murray, H. A., Jr., *J. Biol. Chem.*, 1921, xlv, 233.

<sup>2</sup> Underhill, F. P., and Blatherwick, N. R., *J. Biol. Chem.*, 1914, xviii, 87.

<sup>3</sup> Underhill, F. P., and Blatherwick, N. R., *J. Biol. Chem.*, 1914, xix, 119.

<sup>4</sup> Vosburgh, C. H., and Richards, A. N., *Am. J. Physiol.*, 1903, ix, 35.

after operation." Their protocols agree with their conclusions. The divergence of results appears to demand a reinvestigation of the problem and the results of four experiments are recorded.

#### *Methods.*

Well nourished adult dogs were maintained in a fasting condition throughout the investigation. The operation was performed under morphine-atropine-ether anesthesia, the entire thyroid-parathyroid apparatus being removed. Blood was drawn from an ear vein. Blood sugar estimations were made according to the method of Folin and Wu.<sup>5</sup> Carbon dioxide capacity of the plasma was measured by the procedure of Van Slyke. As a rule determinations on the blood were made about 9.30 a.m. and 5 p.m. daily.

#### *Does Hypoglycemia Occur After Removal of the Thyroids and Parathyroids in Dogs?*

It is quite evident from the data in the table that after complete removal of the thyroids and parathyroids there is a variable but distinct fall in the blood sugar content. This usually occurs after the onset of tetany but may be present before signs of tetany are apparent.

The reason for the difference in the results of Hastings and Murray and our own is not evident unless indeed it is related to the question of nutrition. In all our work with the exception of a single dog the animals were maintained in a fasting state whereas in the investigation of Hastings and Murray the context of their paper would lead one to the conclusion that food was given (*cf.* p. 240). That such an explanation is probably inadequate may be inferred from the fact that in Experiment 3 of our first paper<sup>2</sup> the animal ate food for a period of 12 days after operation, then went into tetany and revealed a condition of hypoglycemia.

With the idea in mind that changes in blood concentration might possibly play a rôle in the topic under discussion hemoglobin and total solid values in the blood were followed. These observations indicate perceptible alterations in concentration from time to time but since these values fluctuate in either direc-

<sup>5</sup> Folin, O., and Wu, H., *J. Biol. Chem.*, 1920, xli, 367.

tion without apparent relationship to tetany or blood sugar content they are without definite significance and hence are omitted here.

The work has now been repeated three times employing three different methods for sugar estimation, and whatever may be the reason for the divergent results we see no occasion for modifying our former conclusion; namely, that removal of the thyroids and parathyroids in dogs induces a condition of lowered blood sugar content.

*The Relation of Alkali Reserve to Removal of the Thyroids and Parathyroids.*

In another section of their communication Hastings and Murray state that after removal of the thyroids and parathyroids there is not the slightest evidence of an alkalosis as indicated by a study of the carbon dioxide capacity of the plasma. This conclusion is directly opposed to the work of Wilson, Stearns, and Thurlow<sup>6</sup> published in 1915. These investigators found by determination of Barcroft's dissociation constant of oxyhemoglobin in venous blood brought into equilibrium with a constant tension of carbon dioxide and measurements of the carbon dioxide tension in the alveolar air evidence of an increasing alkali reserve up to the onset of tetany. McCann<sup>7</sup> later published experiments substantiating this. In view of the importance of the theoretical considerations involved it seemed very desirable to investigate further the problem since the results of Hastings and Murray fail to corroborate the views of Wilson and his colleagues. Advantage has therefore been taken of the opportunity afforded by the animals prepared for the previous work on blood sugar.

From the data presented in the table it must be concluded that up to the onset of tetany little or no change occurs in the alkali reserve of the blood after removal of the thyroids and parathyroids. It is true that with Dog 2 the carbon dioxide capacity is greater the day after the operation than previously. The change is slight, however, and probably little significance should be attached to it, since even greater changes may occur in normal

<sup>6</sup> Wilson, D. W., Stearns, T., and Thurlow, M. De G., *J. Biol. Chem.*, 1915, xxiii, 89.

<sup>7</sup> McCann, W. S., *J. Biol. Chem.*, 1918, xxxv, 553.

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dogs in a fasting condition. This may be seen from data taken from another investigation in which dogs were subjected to a preliminary period of fasting. The following figures are for simple fasting, no other procedure having been followed with the animals concerned.

Days of fasting.....	1	2	3
CO <sub>2</sub> capacity (Dog 1).....	54	68	65
CO <sub>2</sub> " ( " 2).....		51	60

After the onset of tetany in general there may be a decided tendency toward a diminished alkali reserve.

### CONCLUSIONS.

In spite of contrary findings by Hastings and Murray, repetition of previous experiments leads to the reiteration of a former conclusion; namely, that thyroparathyroidectomy results in a lowered blood sugar content.

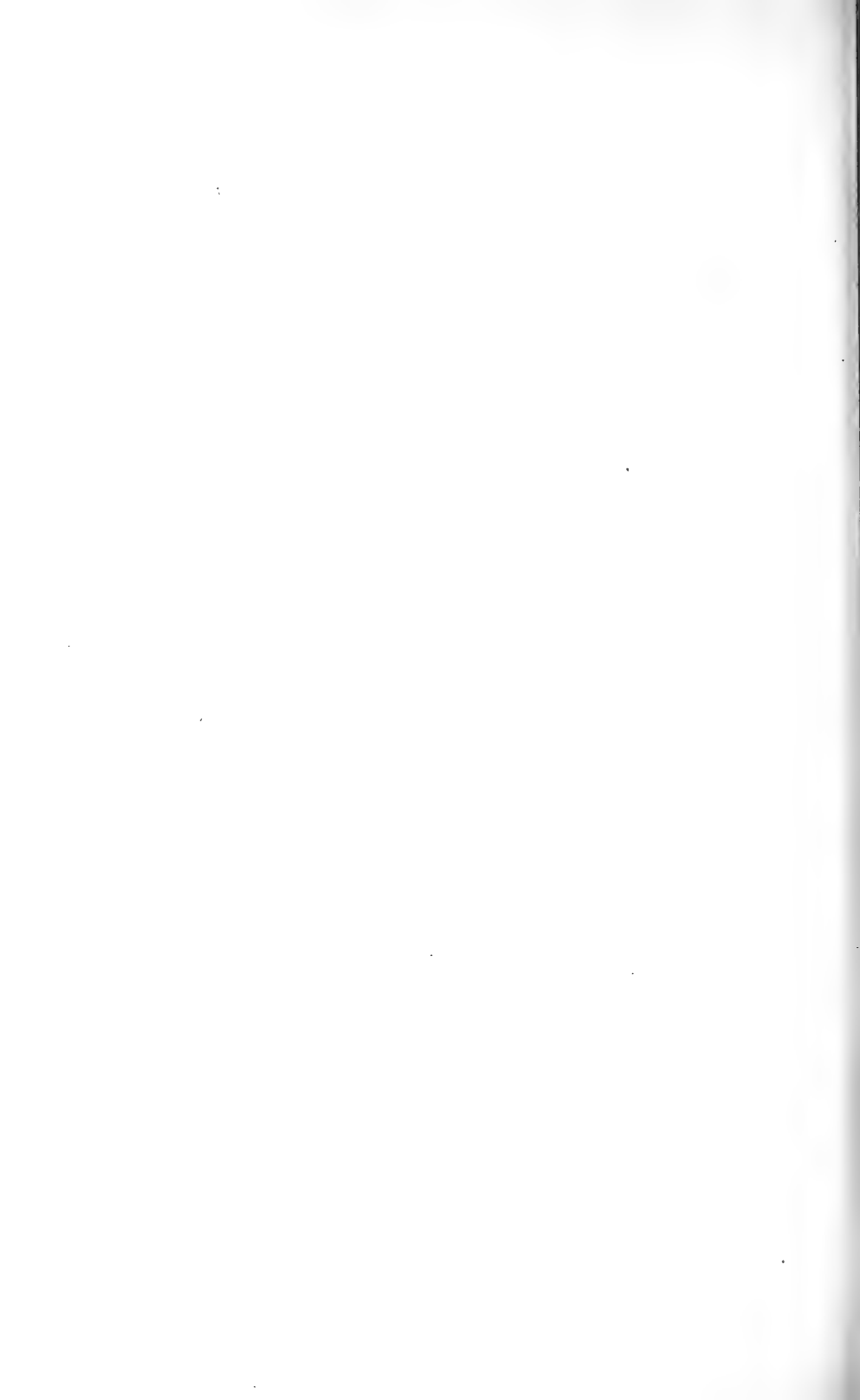
After this operation there seems to be little or no change in the carbon dioxide capacity of the blood up to the onset of tetany. After this period there may be a decided tendency toward a diminished alkali reserve.

### *The Influence of Thyroparathyroidectomy upon Blood Sugar Content and Alkali Reserve.*

Date.	Blood sugar per 100 cc.		Plasma CO <sub>2</sub> capacity.	Remarks.
Dog 1.				
1921	mg.		vol. per cent	
Apr. 20	109	a.m.	70.8 a.m.	Normal.
	111	p.m.	68.9 p.m.	
" 21	111	a.m.		Normal.
" 25	93	"	67.3	"    Removed thyroids and parathyroids 3 p.m. Apr. 25.
" 26	149	"		Appears normal.
	131	p.m.	58.2	
" 27	112	"	55.3	Slight tetany.
" 28	91	a.m.	63.6	
	78	(4.30 p.m.)	58.2	Marked tetany. Died in convulsions at 6.30 p.m.
	58	(5.30 " )	44.7	

*The Influence of Thyroparathyroidectomy—Concluded.*

Date.	Blood sugar per 100 cc.		Plasma CO <sub>2</sub> capacity.	Remarks.
Dog 2.				
1921	mg.		vol. per cent	
May 25	99	p.m.	53.6	Normal.
" 26	98	a.m.	52.9	"      Removed thy- roids and parathyroids at 3 p.m.
" 27	117	"	60.2	Appears normal.
	119	p.m.	58.3	
" 28	45	a.m.	30.9	Severe tetany.
	37	p.m.	44.7	"      "      Died in convulsions.
Dog 3.				
June 6	103	p.m.	60.3	Normal.
" 7	96	a.m.	54.3	"      Removed thy- roids and parathyroids 3 p.m.
" 8	108	"	57.7	Runs, normal.
	101	p.m.	46.4	
" 9	111	a.m.	52.6	
	96	p.m.	48.8	Slight tetany.
" 10	100	(10 a.m.)	54.0	
	101	(11 " )	46.8	Severe tetany.
	67	(10 p.m.)	58.8	
" 11	74	a.m.	49.7	Tetany absent.
	73	p.m.	47.7	Dog comatose.
" 12	85	a.m.	47.7	"      "
	67	p.m.	51.4	
" 13	107	a.m.	51.4	Dog comatose.
	93	p.m.	48.3	
Dog 4.				
June 27	111	a.m.	51.7	Normal. Removal of thy- roids and parathyroids at 3 p.m.
" 28	106	"	49.9	Seems normal.
	100	p.m.	49.9	
" 29	83	a.m.	51.7	Seems normal.
	95	p.m.	49.9	
" 30	81	"	54.1	Slight tetany.
July 1	104	(9.30 a.m.)	42.5	Marked tetany.
	75	(11.30 " )	34.9	"      "
	80	(3.00 p.m.)	32.9	Tetany subsiding.
" 2	94	(8.30 a.m.)	46.8	No tetany.
	96	(Noon.)	46.8	





## THE INFLUENCE OF FOOD INGESTION UPON ENDOGENOUS PURINE METABOLISM. I.

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(Received for publication, August 24, 1921.)

Abundant evidence has been accumulated in recent years to indicate that the original idea of Burian and Schur (1) and Sivén (2), to the effect that the excretion of endogenous purines is constant from day to day in the same individual, is not correct. More than 15 years ago Folin (3) found that the change from a diet of milk and eggs to one of starch and cream might be accompanied by a fall in uric acid elimination of practically 50 per cent, although both diets are purine-free. He stated that the uric acid excretion is reduced whenever the total nitrogen elimination is much diminished, but that the reduction is irregular, and variable for different individuals.

Since the work of Folin, numerous investigators, notably Leathes (4), Mendel and Brown (5), Smetánka (6), Taylor and Rose (7), Mendel and Stehle (8), Lewis and Doisy (9), and Höst (10), have corroborated his findings that protein ingestion exerts a marked influence upon urinary uric acid. Smetánka (6), Mendel and Stehle (8), and Umeda (11) also observed an increase in uric acid elimination, as compared with the fasting output, following the ingestion of carbohydrates. Apparently fats produce the least effect upon purine metabolism of either of the three food-stuffs.

The calorific value of the diet is likewise important in determining the uric acid excretion. According to Graham and Poulton (12), diets of protein and fat of insufficient calorie value, cause a fall of 30 to 50 per cent in the output of endogenous uric acid. If most of the fat is replaced by carbohydrate no fall is observed. More recently Höst (10) has affirmed that every increase or

decrease in the calorific value of the food beyond a certain minimum, is accompanied by a like change in uric acid excretion. This effect occurs no matter which foodstuff is responsible for the variation in energy value of the diet, but is greatest with changes due to protein.

No unanimity of opinion exists, however, as to the cause of the increases and decreases in endogenous uric acid elimination induced by diet. Various factors have been held responsible, and at least six possible explanations have been suggested or advocated in an effort to explain the experimental observations. It is our purpose in this paper to briefly discuss these theories. In the succeeding communication data will be presented which we believe throw additional light on the problem.

Alterations in the excretion of endogenous uric acid resulting from variations in the kind and amount of food have been attributed to the following factors: (1) Nuclear disintegration in the glands of the alimentary canal occasioned by the work of digestion (Mareš (13), Smetánka (6), Lambling and Dubois (14), Mendel and Stehle (8), Höst (10)). (2) Nuclear disintegration associated with the work of digestion and food storage (Smetánka, 6). (3) Synthesis of purines from carbohydrates (Graham and Poulton (12), Umeda (11)). (4) Synthesis of purines from arginine and histidine (Ackroyd and Hopkins (15), Harding and Young (16)). (5) Stimulation of the process of elimination (suggested by Lewis, Dunn, and Doisy (17), but regarded by them as untenable). (6) General stimulation of cellular metabolism by amino-acids or their catabolic derivatives (Lewis, Dunn, and Doisy, 17).

The first of these theories was suggested by Mareš (13). This investigator attributed the increase in output of uric acid following the consumption of purine-free food to nuclear disintegration, chiefly in the alimentary glands, incidental to the physiological work of secretion and digestion. Uric acid thus represents, according to Mareš, the wear and tear of these glandular tissues. When the alimentary glands are resting, the output of uric acid is low; when they are actively synthesizing and secreting digestive fluids, wear and tear is increased, and the production of uric acid is accelerated. As further evidence for this mechanism, Mareš points to the fact that pilocarpine, which is known to increase secretory activity, likewise augments the excretion of uric acid.

Smetánka (6), Lambing and Dubois (14), and Höst (10) also regard digestive work as an important factor in the variations in output of endogenous uric acid. Mendel and Stehle (8), on the other hand, state that their experiments "offer no obstacle to the assumption that *a portion, at least, of the endogenous uric acid may originate from the activity of the alimentary secretory apparatus.*" The latter investigators were able to confirm the findings of Mareš in regard to the action of pilocarpine, and to make the additional observation that atropine, which diminishes secretory activity, causes a fall in uric acid elimination.

The Mareš theory seems inadequate to us for several reasons. It does not appear probable that disintegration of the secretory cells during the process of digestion would be sufficiently extensive to account for the increase in uric acid elimination, which in some case is relatively large (*cf.* Taylor and Rose (7)). It is true that the suggestion of Mendel and Stehle (8) and Höst (10), to the effect that perhaps only a part of the uric acid has its origin in the alimentary cells, obviates this difficulty. Such a suggestion, however, does not afford an explanation of the effect of amino-acids, which require no digestion, upon uric acid elimination. Lewis, Dunn, and Doisy (17) have shown that glycocoll, alanine, and other amino-acids increase the hourly output of uric acid during fasting as much as do proteins. In our own experiments described in the succeeding paper, the protocols show that even though the subjects lived upon weighed diets, necessitating the daily expenditure of the same amount of physiological labor in the process of digestion, the uric acid output for the individual days of the periods was quite variable.

Smetánka (6), who in the main adheres to the Mareš theory, was forced to modify it in view of results obtained by him in experiments somewhat similar in nature to those of Lewis, Dunn, and Doisy. Having observed that the ingestion of honey, a food which like amino-acids requires practically no digestion, causes a marked increase in the output of uric acid, this investigator suggested that in addition to digestive work, the activity involved in glycogenesis may be responsible for a part of the endogenous uric acid. As far as the writer is aware, this is the only suggestion in the literature which specifically attributes uric acid formation in part to the process of food storage. While the theory is

interesting, and perhaps comes nearer explaining the experimental facts than does the original Mareš conception, still it is open to the same criticism as regards the action of amino-acids. If the increased output of uric acid following the ingestion of honey is due to the increased glycogenesis, certainly some other factor must be responsible for the action of glycocoll, alanine, and other compounds which cannot form appreciable quantities of this polysaccharide.

In connection with Smetánka's observation concerning the effect of honey, the investigations of Graham and Poulton (12) and of Umeda (11) are of interest. These authors believe that part of the endogenous uric acid may arise through synthesis from carbohydrates. They observed that carbohydrate-rich fat-poor diets cause a greater excretion of uric acid than do fat-rich carbohydrate-poor diets, even though the protein content and calorific value of the food are maintained constant. Umeda suggests that uric acid may arise from a condensation of urea with an intermediary product of carbohydrate metabolism, perhaps lactic acid. As evidence for a synthesis of purines from carbohydrates, Graham and Poulton point to the observation of Knoop and Windaus (18) that when glucose is exposed *in vitro* to the action of sunlight and the strongly dissociated compound,  $\text{Zn}(\text{OH})_2 \cdot 4\text{NH}_3$ , methyl glyoxal and 5-methyl-imidazole are formed. As interesting as these suggestions are, there exists at the present time no experimental evidence *in vivo* which justifies the belief that carbohydrates are transformed into purines in the animal organism. The observations of Ackroyd and Hopkins (15) which are discussed below indicate that in the rat, at least, purine synthesis from carbohydrate, if it occurs at all, is not quantitatively sufficient to meet the demands of the growing organism for these nuclear constituents.

One of the most important studies of endogenous purine metabolism of recent years is the paper of Ackroyd and Hopkins (15) alluded to above. These authors found that when young rats were supplied diets deprived of arginine and histidine, but adequate in every other respect to meet the demands of growth, growth ceased and the elimination of allantoin decreased 40 to 50 per cent. When either arginine or histidine was present in the diet, there was no loss of weight, and in some cases growth oc-

curred. The decrease in allantoin excretion was likewise much less than when both diamino-acids were absent from the food. No fall in allantoin elimination occurred when tryptophane was removed from the ration, or as a result of the absence of a vitamin supply, though nutritional failure in these cases was even greater than when arginine and histidine were withheld. Despite the difficulties which are obviously associated with metabolic studies involving quantitative urine analyses in small animals, the care with which the experiments of Ackroyd and Hopkins are controlled, and the uniformity of their results, justify, in our opinion, their conclusions that arginine and histidine are the most readily available raw materials for purine anabolism in the body. Apparently either of these amino-acids may serve as the substrate for purine formation.

A similar conclusion as to the origin of purines in the diamino-acids was recently arrived at by Harding and Young (16). According to these investigators, the feeding of placenta, which has a high content of arginine, causes a much greater increase in the output of uric acid and allantoin in young dogs than does the ingestion of an equal quantity of muscle protein. Inasmuch as the diets of their animals were not purine-free, the data of Harding and Young are not as convincing as those of Ackroyd and Hopkins.

On the contrary, Abderhalden and Einbeck (19), Abderhalden, Einbeck, and Schmid (20), and Lewis and Doisy (9) have been unable to show any relationship between the arginine and histidine content of the diet and the uric acid or allantoin output in the urine. Lewis and Doisy compared the effects of diets high and low in arginine and histidine upon the uric acid output in man. Abderhalden and Einbeck studied the effects of adding histidine to the diet upon the allantoin excretion in the dog. In the later experiment of Abderhalden and his coworkers (20), histidine hydrochloride was given in 10 gm. doses to a fasting animal. Neither of the experiments yielded any evidence for an origin of purines in the diamino-acids. We believe the procedures made use of by these investigators were not suitable for studying the relation of amino-acids to purine syntheses. In the experiments of Lewis and Doisy (9), it is quite possible that the "low" histidine-arginine diets contained adequate amounts of purine pre-

cursors to support the normal anabolism. Calculation from the authors' data shows that the "low" diets contained 3.5 to 4.0 gm. of arginine and histidine in each day's ration. If such amounts are adequate (we have no information as to the quantities of diamino-acids required by adult men), one would hardly expect that a more abundant supply would result in an exaggerated purine synthesis, and an increased uric acid elimination. We shall return to this proposition later.

Concerning the experiments of Abderhalden, the question is properly raised by Ackroyd and Hopkins (15) as to whether an abnormal condition like fasting affords the best opportunity for investigating the fate of an amino-acid. They believe<sup>1</sup> that the

" . . . synthesis of such essential tissue constituents as the purines continues during starvation, at the expense—as we are entitled to believe—of protein materials liberated by autolysis of the less essential organs. When however an excess of a single amino-acid enters the circulation of a starving animal in a single isolated dose it may well almost completely escape such special utilization. It appears suddenly in excess of current needs, and, because the processes of deamination and direct oxidation are always in action, it will almost certainly survive for but a short period as available material for synthesis."

In contrast to the methods of Abderhalden and Lewis and their coworkers, Ackroyd and Hopkins compared the effects of diets *free* from arginine and histidine, with diets containing *adequate amounts* of the diamino-acids. The importance of this procedure is emphasized by them as follows:<sup>2</sup>

"When an animal is in a state of full nutrition it does not follow that such a process as the synthesis of the purine ring would necessarily be much accelerated or increased by mere increase in the supply of its raw material."

And again,<sup>2</sup>

"An individual amino-acid fed in excess of the immediate current needs of the tissues, as when it is added to an already efficient dietary, will almost certainly be rapidly broken down on more direct lines, even if it be a normal precursor of the purine (or other) synthesis in the body."

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<sup>1</sup> Ackroyd and Hopkins (15), pp. 552 and 553.

<sup>2</sup> Ackroyd and Hopkins (15), p. 552.

As important as the investigations of Ackroyd and Hopkins appear to us, we do not believe that they, or other studies of their kind, have an immediate bearing upon the problem of the variations in endogenous uric acid elimination incident to alterations in the kind and amount of purine-free food, *when the amino-acids in question are included in the diet*. Even if it be admitted, as we are prepared to do, that tissue purines have their ultimate origin in arginine and histidine, this fact, in our opinion, does not warrant making the assumption that the extent of purine synthesis is proportional to the arginine-histidine supply. On the contrary, it seems reasonable to suppose that the anabolism of any tissue component is limited quantitatively to the needs of the organism for that particular ingredient. As soon as a diet contains sufficient precursors of any given anabolic product, synthesis of that product at the optimum rate probably occurs. It seems unlikely that the optimum would be exceeded however great a redundancy of the precursors in question were provided. We believe that this view is entirely in accord with the statements of Ackroyd and Hopkins quoted above, and is completely justified in the case of purine anabolism by the experiments of Abderhalden and Lewis and Doisy. If we are correct, one should no more expect to exaggerate purine anabolism by feeding excessive quantities of purine precursors, than he should anticipate being able to increase the mass of brain substance by feeding unusual amounts of the components of nervous tissue. With the exception of the purely storage forms of foods (glycogen, fats, and to a less extent, amino-acids), the components of the tissues of each species are normally synthesized and retained in remarkably uniform proportions. If conditions were otherwise, tissue composition would be largely determined by the accident of diet rather than by the expression of the inherent, hereditary tendencies and impulses of the organism. It is, therefore, rather surprising to us that Harding and Young (16) were able to note differences in purine excretion in pups on diets of placenta (high in diamino-acids) as contrasted with diets of muscle (low in diamino-acids), unless the differences were in part due to exogenous purines. On the other hand, the fact that in their experiments growing animals were used, in which the anabolic reactions are known to predominate, may have been responsible for their unique data.

In accordance with these concepts, *instead of there being conflict between the data of Ackroyd and Hopkins on the one hand, and Lewis and Doisy on the other, we regard them as entirely in accord and mutually supplementary.* In the experiments of the former, the *decrease* in allantoin excretion following the removal of arginine and histidine from the diet is the significant point, rather than the increase, which probably represented the normal purine metabolism, when the diamino-acids were supplied. After removal of arginine and histidine from the diet growth ceased because purine (and perhaps other) anabolism was no longer possible. Because of the deficient anabolism, greater physiological economy was exercised in catabolism, and the catabolic end-product of purines in the rat (allantoin) decreased in amount. In the experiments of Lewis and Doisy, a high arginine-histidine diet *failed to induce a greater elimination* of the catabolic end-product of purines in man (uric acid) than did a low arginine-histidine diet, because the latter was adequate to permit the optimum anabolism of purines. The superfluous molecules of arginine and histidine were doubtless oxidized without passing through the purine stage. In other words, the work of Ackroyd and Hopkins, to our mind, renders it very probable that the ultimate sources of tissue purines are arginine and histidine; the investigation of Lewis and Doisy indicates that purine anabolism in the adult is limited in extent to the physiological needs of the organism for purines. Neither investigation, however, permits any conclusions to be drawn as to the cause of variations in purine elimination with diets containing adequate amounts of diamino-acids. The latter problem is more likely one of purine catabolism or excretion rather than of anabolism.

In the course of the exceedingly interesting investigation of Lewis, Dunn, and Doisy (17) on the influence of diet upon the hourly elimination of uric acid, the possibility occurred to the authors that the increased uric acid excretion following the ingestion of a single dose of a protein or of an amino-acid might be due to a *stimulation of the processes of excretion* under the influence of the food, rather than to increased uric acid formation. They reasoned that if a single dose of an amino-acid produced its effect by bringing about the mobilization and elimination of reserve or stored purines or their precursors, the administration of a



second dose, after the effect of the first had reached its maximum, should be without further influence. Accordingly, an experiment was made in which successive doses of glycocholate were administered on the same experimental day. The figures show that entirely comparable increases in the hourly output of uric acid occurred after each dose. According to the authors the data<sup>3</sup> "clearly demonstrate that the effects of amino-acids on uric acid excretion are not the result of stimulation of excretory processes leading to a removal of preformed uric acid from the body." While it might be questioned whether Lewis, Dunn, and Doisy were justified in assuming that the first dose of glycocholate entirely removed *all* excess or reserve purines from the system, and whether the single experiment reported by them is sufficient to warrant their conclusion in this regard, data of another sort in the literature, when considered in connection with their work, increase the probability of their contention being well founded. Frequent estimations of uric acid in the blood of normal subjects upon widely different purine-free diets led Höst (10) to the conclusion that diet (in the absence of purines) is without influence upon the concentration of uric acid in the blood. Despite the fact that urinary uric acid varies greatly in a given individual as a result of changes in the composition of the food, the proportion in the blood remains constant within the experimental error of the method. Inasmuch as uricolysis is not believed to occur in the human subject, and since the concentration of uric acid in normal blood is invariable under the influence of purine-free food, Höst is of the opinion<sup>4</sup> that "the endogenous uric acid output becomes a direct expression for the uric acid formation." It must be admitted that rather large quantities of uric acid would have to be retained in the blood in order to alter appreciably the proportion present, or that reserve purines in the sense of Lewis, Dunn, and Doisy might be stored in the tissues, and hence not be manifested by blood analyses at all. Nevertheless, such evidence as we have, whether obtained from a study of the urine (Lewis, Dunn, and Doisy), or by means of blood analyses (Höst), indicates that the cause of the alterations in output of endogenous

<sup>3</sup> Lewis, Dunn, and Doisy (17), p. 17.

<sup>4</sup> Höst (10), p. 30.

uric acid following food consumption is not to be sought in an exaggerated excretion.

Having excluded to their own satisfaction the possibility of a stimulation in excretion being the causative factor in the increased output of uric acid following protein ingestion, Lewis, Dunn, and Doisy (17) suggest that the effect may be due to a general stimulation of all cellular metabolism by amino-acids. Each of the four amino-acids, glycocoll, alanine, glutaminic acid, and aspartic acid, as well as the closely related asparagine, caused an appreciable increase in the hourly fasting output of uric acid. The stimulation caused by the dicarboxylic amino-acids was more marked than that produced by glycocoll and alanine. On the other hand, sarcosine, a substituted amino-acid not readily catabolized by the body, and ammonium chloride and urea, were without influence. The authors emphasize the similarity of the effects produced by protein and amino-acid ingestion upon uric acid formation and heat production (specific dynamic action), and point out that the same chemical factors may be responsible for both.

We believe that there are no experiments in the literature the results of which invalidate the assumption that general stimulation of cellular catabolism, involving both the nuclear purines and the hypoxanthine of muscle tissue, by amino-acids is at least one of the important factors in endogenous purine metabolism. Particularly do the data of Smetánka (6), Taylor and Rose (7), Mendel and Stehle (8), and Höst (10) lend support to this hypothesis. In the succeeding paper we shall present the results of observations which we believe afford additional reasons for accepting this view.

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## THE INFLUENCE OF FOOD INGESTION UPON ENDOGENOUS PURINE METABOLISM. II.

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(Received for publication, August 24, 1921.)

The investigations reported in this communication have been in progress recurrently for the past 5 years. At first we made numerous studies of the hourly elimination of uric acid in fasting, and following the ingestion of single meals of purine-free foods. The results obtained were, for the most part, similar to those of other investigators who have studied uric acid excretion during short periods, notably, Mendel and Stehle (1), Neuwirth (2), and Lewis, Dunn, and Doisy (3). Invariably, the consumption of food led to an increase in uric acid excretion. But despite every care in the conduct of the experiments, the increases were sometimes so slight, and the fluctuations without the influence of food were relatively so large, that we regarded the interpretation of the data as liable to serious error. We finally abandoned hourly studies and adopted 24 hour periods, which yielded more consistent results.

### *Methods.*

The plan of the investigation was similar to that pursued in a study of endogenous creatine-creatinine metabolism, the results of which were reported in a former communication (4). In the earlier experiments, the uric acid and total nitrogen excretion were determined in subjects ingesting diets alternately low and high in protein. Later, two additional series of studies were undertaken. In one series, the nitrogen intake was maintained constant throughout, while the calorific value of the diet was altered. In the other, a low protein-low calorific diet was alternated with a high protein-high calorific diet.

The subjects were four healthy young men, students in the School of Medicine of this University, who were engaged in the usual routine of student life, with practically the same amount of physical activity each day throughout the experiments. By making use of several individuals, we have excluded the possibility of our findings being accidental, or due to metabolic peculiarities of a single subject. Each student had been subsisting upon a purine-free diet for several days preceding the experimental régime. The diets were prepared, weighed, and ingested in the laboratory, and the meals were served at regular hours three times daily. Urines were preserved with toluene, and were usually

TABLE I.  
*Composition of Diets.*

No. of diet.	Articles of diet.							Food values.*	
	Bread.	Honey.	Butter.	Eggs.	Milk.	Cheese.	Apples.	N	Calories.
	gm.	gm.	gm.	gm.	cc.	gm.	gm.	gm.	
1	400	150	150					6.17	2,770
2	150			655	1,500	50		27.93	2,741
7	400		20	200		50		12.91	1,780
8	400	75	150	100	790		300	12.86	3,433
9	300	150	60				300	4.75	1,978
10	300		90	450	1,500	90	300	27.45	3,907

\* The nitrogen and calorific values here tabulated were calculated from data given by Atwater, W. O., and Bryant, A. P., *U. S. Dept. Agric., Office Exp. Stations, Bull. 28* (revised), 1906.

analyzed immediately after the end of the 24 hour periods. Total nitrogen was estimated by the Kjeldahl-Gunning method, and uric acid by the procedure of Benedict and Hitchcock (5). Table I indicates the composition, nitrogen content, and calorific value of each diet, all of which were of course practically purine-free.<sup>1</sup>

<sup>1</sup> White bread, which contains larger traces of purines than any other article of food included in the diets, was kept constant in quantity throughout each experiment with the exception of those recorded in Tables II and III. In these two, more bread was ingested during the low than during the high diet periods. Accordingly, the effect of traces of exogenous purines, if appreciable at all, would be more pronounced during the periods of low diet, and therefore cannot impair the validity of our conclusions.

## EXPERIMENTAL.

The results of the numerous experiments which we have conducted are entirely in accord with each other, and therefore need not all be detailed in this communication. The protocols give

TABLE II.

*The Influence of High and Low Protein Diets on Uric Acid Excretion, when the Calorific Intake is Constant.*

Experiment 8. Subject F. W. D.

Date.	Body weight.	Urine volume.	Reaction to litmus.	Total N.	Uric acid.	Remarks.
1916	kg.	cc.		gm.	gm.	
Dec. 7	72.5	1,310	Acid.	6.97	0.40	Low protein diet, No. 1. 6.17 gm. N and 2,770 calories daily.*
" 8		1,550	"	7.38	0.47	
" 9		1,510	"	7.12	0.41	
" 10		2,450	"	7.96	0.44	
" 11		1,180	"	6.78	0.43	
Average.....				7.24	0.43	
Dec. 12	71.6	1,675	Acid.	13.00	0.51	High protein diet, No. 2. 27.93 gm. N and 2,741 calories daily.
" 13		2,390	"	16.49	0.49	
" 14		2,780	"	20.49	0.46	
" 15		2,440	"	22.00	0.51	
" 16		2,690	"	22.78	0.48	
Average.....				18.95	0.49	
Dec. 17	71.6	2,765	Acid.	15.06	0.40	Low protein diet, No. 1. 6.17 gm. N and 2,770 calories daily.
" 18		1,340	"	9.91	0.39	
" 19		1,400	"	8.74	0.39	
Average.....				11.24	0.39	
1917						
Jan. 18	71.6	1,530	Acid.	9.03	0.29	Starvation level.†

\* Began eating this diet on Dec. 5.

† Subject ate a purine-free diet for 10 days, followed by a fast of 40 hours. Analyses represent urine of last 24 hours of the fast.

the data of six experiments, two of each of the three series indicated above. In Tables II and III are shown the effects of alternately feeding low protein and high protein diets when the calorific intake is maintained constant. Although there is some

irregularity in the uric acid elimination, as is almost invariably the case with this urine constituent, nevertheless, there is a slight but unmistakable increase in output during the high protein periods. The return to the low protein diet, following the days

TABLE III.

*The Influence of High and Low Protein Diets on Uric Acid Excretion, when the Calorific Intake is Constant.*

Experiment 9. Subject H. L. B.

Date.	Body weight.	Urine volume.	Reaction to litmus.	Total N.	Uric acid.	Remarks.
1916	kg.	cc.		gm.	gm.	
Dec. 7	68.6	400	Acid.	6.73	0.45	Low protein diet, No. 1. 6.17 gm. N and 2,770 calories daily.*
" 8		460	"	6.96	0.51	
" 9		450	"	6.62	0.54	
" 10		730	"	7.26	0.56	
" 11		520	"	6.42	0.54	
Average.....				6.80	0.52	
Dec. 12	68.8	980	Acid.	12.45	0.66	High protein diet, No. 2. 27.93 gm. N and 2,741 calories daily.
" 13		960	"	16.33	0.56	
" 14		980	"	19.02	0.52	
" 15		1,160	"	21.74	0.54	
" 16		1,100	"	22.18	0.55	
Average.....				18.35	0.57	
Dec. 17	69.1	635	Acid.	12.89	0.47	Low protein diet, No. 1. 6.17 gm. N and 2,770 calories daily.
" 18		460	"	8.86	0.48	
" 19		450	"	8.21	0.46	
Average.....				9.99	0.47	
1917						
Jan. 18	69.5	710	Acid.	9.36	0.43	Starvation level.†

\* Began eating this diet on Dec. 5.

† Subject ate a purine-free diet for 10 days, followed by a fast of 40 hours. Analyses represent urine of last 24 hours of the fast.

of high protein ingestion, is in each case accompanied by a fall in uric acid excretion. At the bottom of each table is recorded the subject's fasting output of uric acid. As indicated in the protocols, purine-free diets were ingested for 10 days preceding



the 40 hour fasts. The urines for the last 24 hours of the abstinence periods were used for the analyses. In Experiment 8, the starvation uric acid excretion dropped to 0.29 gm., and in Experiment 9, to 0.43 gm. per day.

TABLE IV.

*The Influence on Uric Acid Excretion of Diets High and Low in Calories, when the Protein Intake is Constant.*

Experiment 15. Subject J. S. D.

Date.	Body weight.	Urine volume.	Reaction to litmus.	Total N.	Uric acid.	Remarks.
1917	kg.	cc.		gm.	gm.	
Dec. 7	62.2	685	Acid.	11.63	0.41	Low calorific diet, No. 7. 12.91 gm. N and 1,780 calories daily.*
" 8		865	"	13.33	0.47	
" 9		1,000	"	12.35	0.49	
" 10		560	"	11.66	0.47	
" 11		615	"	12.56	0.47	
" 12		620	"	12.20	0.54	
Average.....				12.29	0.48	
Dec. 13	61.4	625	Acid.	11.15	0.50	High calorific diet, No. 8. 12.86 gm. N and 3,433 calories daily.
" 14		825	"	11.21	0.58	
" 15		1,490	"	11.17	0.59	
" 16		800	"	9.16	0.55	
Average.....				10.67	0.56	
Dec. 17	61.5	650	Acid.	10.84	0.55	Low calorific diet, No. 7. plus 300 gm. apples. <sup>†</sup> 13.10 gm. N and 1,971 calories daily.
" 18		890	"	12.30	0.56	
" 19		995	"	12.35	0.57	
" 20		1,175	"	11.93	0.57	
Average.....				11.86	0.56	

\* Began eating this diet on Dec. 3.

† The apples were added because of their laxative properties.

Tables IV and V show the effects of alternately feeding diets low and high in calories when the protein intake is maintained practically constant. Throughout each experiment the nitrogen consumption was approximately 13.0 gm. (12.86 to 13.10 gm.) daily. In the first and third periods the energy values of the diets were 1,780 and 1,971 calories respectively. In the second

or high calorific period, an increase to 3,433 calories daily was made by the liberal addition of carbohydrates and fats. The data indicate that in each experiment the change to the ration of greater calorific value was accompanied by a rise in uric acid elimination. In Experiment 15, the increase was from an average

TABLE V.

*The Influence on Uric Acid Excretion of Diets High and Low in Calories, when the Protein Intake is Constant.*

Experiment 16. Subject J. B. F.

Date.	Body weight.	Urine volume.	Reaction to litmus.	Total N.	Uric acid.	Remarks.
1917	kg.	cc.		gm.	gm.	
Dec. 7	65.2	975	Acid.	11.81	0.42	Low calorific diet, No. 7. 12.91 gm. N and 1,780 calories daily.*
" 8		840	"	11.48	0.45	
" 9		880	"	12.96	0.44	
" 10		1,070	"	12.60	0.48	
" 11		920	"	13.00	0.58	
" 12		1,040	"	12.26	0.52	
Average.....				12.35	0.48	
Dec. 13	64.3	720	Acid.	11.51	0.57	High calorific diet, No. 8. 12.86 gm. N and 3,433 calories daily.
" 14		730	"	10.21	0.59	
" 15		1,485	"	10.69	0.64	
" 16		1,270	"	9.26	0.55	
Average.....				10.42	0.59	
Dec. 17	65.5	1,360	Acid.	10.67	0.59	Low calorific diet, No. 7, plus 300 gm. apples.† 13.10 gm. N and 1,971 calories daily.
" 18		1,040	"	10.96	0.52	
" 19		920	"	11.68	0.59	
" 20		850	"	11.92	0.62	
Average.....				11.31	0.58	

\* Began eating this diet on Dec. 3.

† The apples were added because of their laxative properties.

of 0.48 gm. daily in the low period, to an average of 0.56 gm. in the high period. In Experiment 16, the increase was quantitatively similar. In each experiment the rise in uric acid elimination amounted to 17 to 18 per cent. A third investigation upon another subject gave results entirely comparable in every particular to the two here reported.

In each experiment of this type, as contrasted with those in which the energy value of the food was maintained constant while the protein intake was varied (Tables II and III), there was no decrease in uric acid elimination during the 4 days of the period in which a return was made to the low calorific diet. Uric acid continued to be excreted at the higher level established upon the diet of greater energy value. Perhaps the storage of carbohydrates and fats upon the high calorie diet was sufficiently large to supply an abundance of energy-yielding food material during the after period, and thus temporarily prevent the effects of a return to the low ration becoming apparent in the uric acid excretion. We did not determine how long this condition would persist before the output would again decrease to the low diet level.

Experiments 19 and 20 are illustrative of the effects brought about by alternately feeding low protein-low calorific and high protein-high calorific diets. Each of these experiments was continued over four periods—two upon the low and two upon the high ration—so that each increase and decrease in uric acid elimination is reproduced a second time. The data are given in Tables VI and VII. The food consumption was identical in both experiments. The low ration consisted of 4.75 gm. of nitrogen and 1,978 calories per day; the high, of 27.45 gm. of nitrogen and 3,907 calories per day. As shown in the tables, the change from a ration low in protein and calories to one having a high protein and calorific content is in every instance attended by an increase in uric acid excretion. On certain days this is relatively quite large, as for example on the 15th, 16th, and 27th in each experiment. On the latter date the urines of both subjects contained rather large quantities of uric acid crystals. Likewise, the change from a high to a low diet is associated with a prompt and distinct drop in uric acid elimination, as for instance on the 22nd in each experiment.

The averages for the periods manifest an unmistakable relationship between uric acid excretion and the character of the food, but we believe the figures for the individual days are more instructive than are the averages. A study of the data indicates that the most decided alterations in uric acid output associated with changes in the quantity of protein, occur almost invariably on

TABLE VI.

*The Influence of a High Protein-High Calorific Diet and a Low Protein-Low Calorific Diet on Uric Acid Excretion.*

Experiment 19. Subject H. L. B.

Date.	Body weight.	Urine volume.	Reaction to litmus.	Total N.	Uric acid.	Remarks.
1918	kg.	cc.		gm.	gm.	
Nov. 11	66.4	680	Acid.	5.08	0.44	Low protein-low calorific diet, No. 9. 4.75 gm. N and 1,978 calories daily.*
" 12		670	"	4.37	0.46	
" 13		670	"	5.06	0.43	
" 14		680	"	4.96	0.43	
Average.....				4.87	0.44	
Nov. 15	65.9	960	Acid.	12.56	0.55	High protein-high calorific diet, No. 10. 27.45 gm. N and 3,907 calories daily.
" 16		1,160	"	12.92	0.58	
" 17		1,420	"	18.17	0.49	
" 18		1,610	"	19.08	0.51	
" 19		1,400	"	20.08	0.51	
" 20		1,230	"	20.79	0.54	
" 21		1,470	"	21.34	0.56	
Average.....				17.85	0.53	
Nov. 22	68.2	1,490	Acid.	12.56	0.42	Low protein-low calorific diet, No. 9. 4.75 gm. N and 1,978 calories daily.
" 23		840	"	8.82	0.43	
" 24		820	"	7.22	0.42	
" 25		800	"	7.69	0.49	
" 26		710	"	7.14	0.58	
Average.....				8.69	0.47	
Nov. 27	66.4	1,030	Acid.	15.96	0.71†	High protein-high calorific diet No. 10. 27.45 gm. N and 3,907 calories daily.
" 28		1,220	"	18.54	0.53	
" 29		1,240	"	18.73	0.49	
" 30		1,360	"	25.18	0.49	
Average.....				19.60	0.56	

\* Began eating this diet on Nov. 4.

† Urine contained a deposit of uric acid crystals on this day.

the first day after the inauguration of the dietary changes. After the initial maximum variations, there appears to be a general tendency to gradual recovery from the effects of the sudden alteration in type of food. This is particularly noticeable in the

TABLE VII.

*The Influence of a High Protein-High Calorific Diet, and a Low Protein-Low Calorific Diet on Uric Acid Excretion.*

Subject J. S. D.

Date.	Body weight.	Urine volume.	Reaction to litmus.	Total N.	Uric acid.	Remarks.
1918	kg.	cc.		gm.	gm.	
Nov. 11	60.2	1,070	Acid.	4.75	0.42	Low protein-low calorific diet, No. 9. 4.75 gm. N and 1,978 calories daily.*
" 12		600	"	4.41	0.41	
" 13		1,080	"	5.42	0.49	
" 14		700	"	4.12	0.39	
Average.....				4.68	0.43	
Nov. 15	60.0	1,380	Acid.	13.23	0.67	High protein-high calorific diet, No. 10. 27.45 gm. N and 3,907 calories daily.
" 16		1,830	"	16.21	0.59	
" 17		2,250	"	16.52	0.50	
" 18		1,200	"	17.28	0.47	
" 19		1,190	"	19.07	0.51	
" 20		1,310	"	21.13	0.52	
" 21		1,470	"	21.67	0.56	
Average.....				17.87	0.55	
Nov. 22	61.6	1,595	Acid.	12.19	0.37	Low protein-low calorific diet, No. 9. 4.75 gm. N and 1,978 calories daily.
" 23		500	"	7.48	0.34	
" 24		730	"	7.48	0.33	
" 25		590	"	6.34	0.39	
" 26		998	"	6.85	0.44	
Average.....				8.07	0.37	
Nov. 27	59.8	810	Acid.	12.01	0.66†	High protein-high calorific diet, No. 10. 27.45 gm. N and 3,907 calories daily.
" 28		1,750	"	18.09	0.55	
" 29		1,740	"	17.44	0.52	
" 30		2,020	"	21.49	0.46	
Average.....				17.26	0.55	

\* Began eating this diet on Nov. 4.

† Urine contained a deposit of uric acid crystals on this day.

last periods of Experiments 19 and 20. In Experiment 19, the uric acid figures for the 4 days of the final period are 0.71, 0.53, 0.49, and 0.49 gm. respectively, showing that the effect upon uric acid became less pronounced the longer the diet was ingested.

Experiment 20 manifests a similar behavior. In this subject, the figures for uric acid for the last 4 days of high diet are 0.66, 0.55, 0.52, and 0.46 gm. respectively.

Furthermore, the changes in uric acid excretion are more marked during the latter half than during the first half of Experiments 19 and 20. Apparently the influence became exaggerated in these experiments in proportion to the frequency with which the alterations in diet were instituted. Thus the increase from the average output of the first period to the first day of the second period, is not so pronounced as is the increase from the average elimination of the third period to the first day of the fourth period. The latter amounts to 51 and 78 per cent respectively in the two experiments.

While the actual quantities of "extra" uric acid are not large in any of the experiments, yet in proportion to the normal output, the changes are appreciable, and in our opinion, leave no room for doubt as to the influence of food upon endogenous purine metabolism. In none of the experiments is there any proportionality between the volume of urine and the quantity of uric acid excreted.

#### DISCUSSION.

The interpretation of our results is fraught with difficulty because of unavoidable irregularities, as noted above, in the uric acid data. The following features, however, warrant special comment. (a) An increase in the consumption of purine-free food, either in the form of protein or as non-nitrogenous articles of diet, leads to a small but distinct increase in the daily output of uric acid. This is in accord with the findings of other investigators, notably Mareš (6), Leathes (7), Smetánka (8), Taylor and Rose (9), Mendel and Stehle (1), Lewis and Doisy (10), and Höst (11). (b) Under the conditions of our experiments, the maximum effect upon uric acid excretion produced by an increase in protein consumption, usually manifests itself upon the first day after the inauguration of the dietary change, and in some cases is followed by a tendency to return to a lower level of elimination with continued use of the high protein ration. (c) Increases or decreases in endogenous uric acid excretion resulting from abrupt alterations in food consumption, seemingly be-

come more pronounced the more frequently the changes in diet are instituted (*cf.* Tables VI and VII).

The above results are in some respects quite different from what we had expected to obtain. In the single experiment reported several years ago by Taylor and Rose (9), the uric acid elimination steadily increased upon a high protein diet until it amounted to 0.82 gm. per day, as contrasted with an average of 0.29 gm. for the low protein period. At no time did the uric acid excretion manifest a tendency to decrease, but on the contrary progressively increased upon the high protein ration. We therefore anticipated that similar effects would be observed in the experiments described in the present communication. Probably the differences are to be accounted for in the quantity of food ingested. In the experiment of Taylor and Rose, an accurate record of food consumption was not attempted, but the subject, for a period of 4 days, ingested as heavily of white of egg as possible. The nitrogen consumption each day amounted to over 40 gm. Furthermore, in the experiment of Taylor and Rose, the diet of the fore period consisted of purified starch and cane-sugar, and hence was nitrogen-free. The abrupt change from such a diet to one containing an excess of 40 gm. of nitrogen per day, was a far more radical dietary alteration than any which our subjects experienced in the present investigation.

In attempting to explain the effects of protein upon uric acid excretion, we are forced to a conclusion similar to that of Lewis, Dunn, and Doisy (3); namely, that at least one of the factors involved is a *general stimulation of cellular metabolism by amino-acids*. For reasons stated in the preceding paper, we do not believe that this stimulating effect is limited to the digestive glands, or that the increases in uric acid are due to digestive work. If physiological labor is to be held responsible for the effect of diet upon uric acid, it would seem more reasonable to include as contributing factors all of the activities involved in digesting, storing, and metabolizing the foods, rather than to single out one group of activities, as is done in the Mareš theory, and attach the responsibility solely to them. We are convinced, however, that physiological activity *per se* is not the important factor. The work involved in metabolizing the comparatively small doses of amino-acids fed in the experiments of Lewis, Dunn,

and Doisy would scarcely have necessitated sufficient cellular wear and tear to account for the increases in uric acid elimination observed by these authors. We believe that a more logical explanation is to be sought in a general accelerating action of amino-acids upon the metabolic processes. Why in our experiments this should have apparently become more pronounced the more frequently the changes in diet were instituted, is not evident.

Lewis and his collaborators (3) call attention to the fact that probably the causal agents for the cellular stimulation are "either the amino-acids or their non-nitrogenous rest,  $\alpha$ -ketonic or hydroxy acids."<sup>2</sup> Certain facts in our experiments may be of interest in this connection. In spite of irregularities, the data in Tables VI and VII evidence a general tendency for the uric acid output to be greatest when the temporary retention of nitrogen is most pronounced, and to fall to lower levels when the nitrogen of the food is promptly excreted. In other words, during a condition of plus-balance, uric acid excretion is usually increased. When a state of minus-balance pertains, as for a few days following the change from a high protein to a low protein ration, the elimination of uric acid tends to decrease. We know from the work of Mendel and R. C. Lewis (12) and others, that urea nitrogen, in normal individuals, is promptly excreted. On the other hand, Folin and Denis (13), and Van Slyke and Meyer (14) have shown that amino-acids may be temporarily stored unchanged in the tissues. Folin and Denis (13) make the following interesting comment concerning their important discovery:<sup>3</sup>

"The muscles and other tissues as well evidently serve as a storehouse for such reserve materials [amino-acids]. The existence of such a reservoir must be taken into account in our theories of protein metabolism. . . . The peculiar lag extending over several days in the establishment of a constant level of nitrogen elimination when extreme changes are made in the nitrogen intake is probably due to a filling or depletion as the case may be of the reservoir."

It would thus seem reasonable to assume that in our experiments retained nitrogen was in the form of amino-acids, which were being used to fill the "reservoir." But during the process

<sup>2</sup> Lewis, Dunn, and Doisy (3), p. 25.

<sup>3</sup> Folin and Denis (13), pp. 94 and 95.



of "filling," uric acid excretion was at a higher level than during the course of "depletion." May this not indicate that unchanged amino-acids, rather than their disintegration products, are responsible for the stimulating effect upon cellular metabolism?

In Chart 1 are shown curves of the uric acid excretion and the state of nitrogen equilibrium in Experiment 19, Table VI. The curve of nitrogen balance is not strictly correct since we have no data upon alimentary nitrogen loss. Despite this fact, and the irregularities in the uric acid figures, a general interrelationship is manifested. It must be recalled that uric acid is probably the most difficult of the urinary ingredients for the kidneys to excrete, and that accordingly, irregularities in elimination and temporary retentions are more likely to occur in the case of it than of any other metabolic product. Hence a close agreement between the curves would hardly be expected. Experiments 8, 9, and 20 (Tables II, III, and VII) show a similar general proportionality between the uric acid output and the nitrogen balance.

In regard to the increased excretion of uric acid in Experiments 15 and 16 (Tables IV and V), where the protein consumption was constant, but the calorific values of the diets varied, the interpretation is more difficult. Similar effects of increases in the caloric value of the food have been observed by Höst (11). The protein-sparing action of carbohydrates is indicated in the second period of each of our experiments by a fall in the output of total nitrogen. Doubtless the retained amino-acids were partly responsible for the rise in uric acid, through their stimulating action upon cellular catabolism. It is also quite possible that metabolism may be accelerated by intermediary products derived from carbohydrates and fats. We know that these food-stuffs do manifest a stimulating effect upon heat production, but to a more limited extent than do proteins.

Another factor which we believe should be taken into consideration in a discussion of endogenous purine metabolism, is the possibility of a reutilization of the purines liberated in catabolism. We are in the habit of thinking of these purines as being transformed into uric acid and with more or less promptness eliminated. On the other hand, in starvation and in other conditions of physiological stress, unusual economy may be exercised in metabolism.

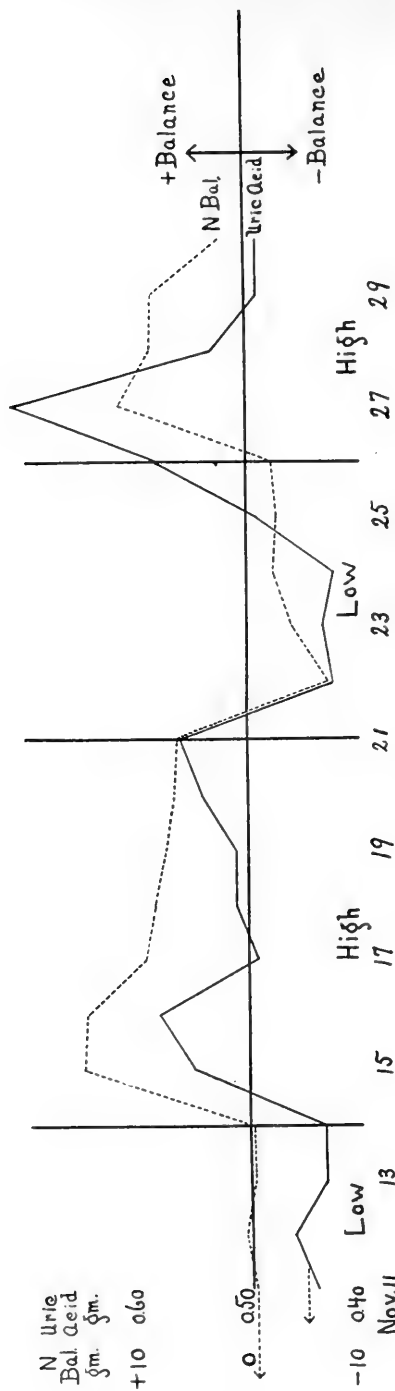


CHART 1. Experiment 19. Subject H. L. B.

May it not be possible, when there is a deficiency of purine precursors (arginine and histidine?; *cf.* Ackroyd and Hopkins (15), and Harding and Young (16)) in the diet, that a reutilization of purines liberated in tissue wear and tear may occur for synthetic purposes? Such an assumption would account for the remarkably low figures for uric acid excretion observed by Raiziss, Dubin, and Ringer (17) in individuals living upon starch-cream diets. Reutilization might also have been a contributing factor in producing the low values which we obtained during brief fasts (Tables II and III). Under such conditions urinary uric acid would represent a balance between the formation and conservation of purines.

A consideration of all the known facts concerning purine metabolism, both those discussed in the preceding paper, as well as the experimental data presented in this communication, appears to indicate that endogenous purines have their ultimate origin in arginine and histidine, but that the extent of their synthesis is limited quantitatively to the anabolic needs of the organism. Superfluous molecules of arginine and histidine, which are not required for anabolism, are probably in the adult at least oxidized without preliminary transformation into purines. Under conditions of constant diet and nitrogen equilibrium, purine metabolism, as measured by the uric acid output, proceeds at a fairly constant rate, but this rate may be altered by changes in the character or quantity of food ingested. Amino-acids and probably digestive (or metabolic) products of carbohydrates and fats, exert a general stimulating action upon cellular catabolism, which is manifested by a rise in uric acid elimination following marked increases in food consumption. Moreover, indirect evidence indicates that perhaps in the case of the amino-acids, they themselves, rather than their nitrogen-free derivatives, are the stimulating agents. It is suggested that when the organism is deprived of purine precursors, an additional factor leading to variations in uric acid excretion, may be a reutilization for anabolic purposes of part of the purines liberated in catabolism.

Such a working hypothesis, while wholly tentative, serves for the present to explain many apparent contradictions in the literature. We expect to test the possibility of a reutilization of purines in subsequent studies.

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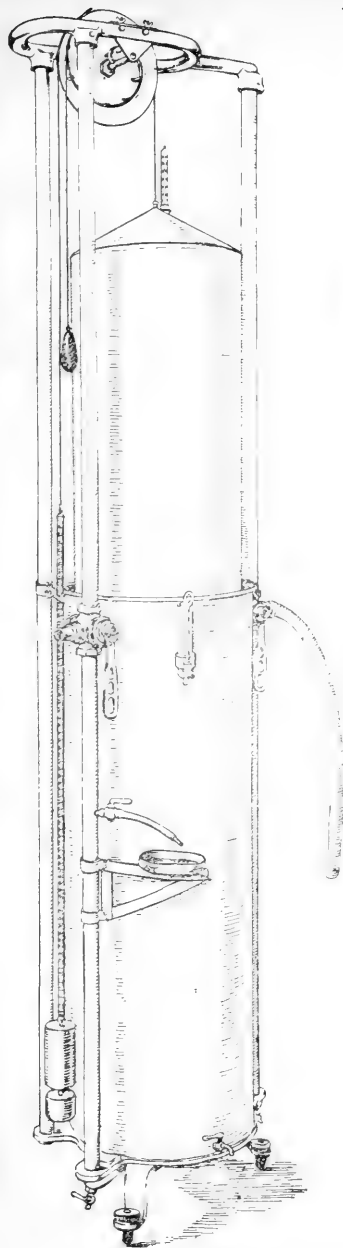
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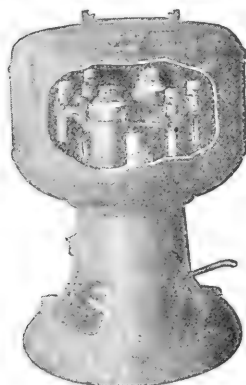
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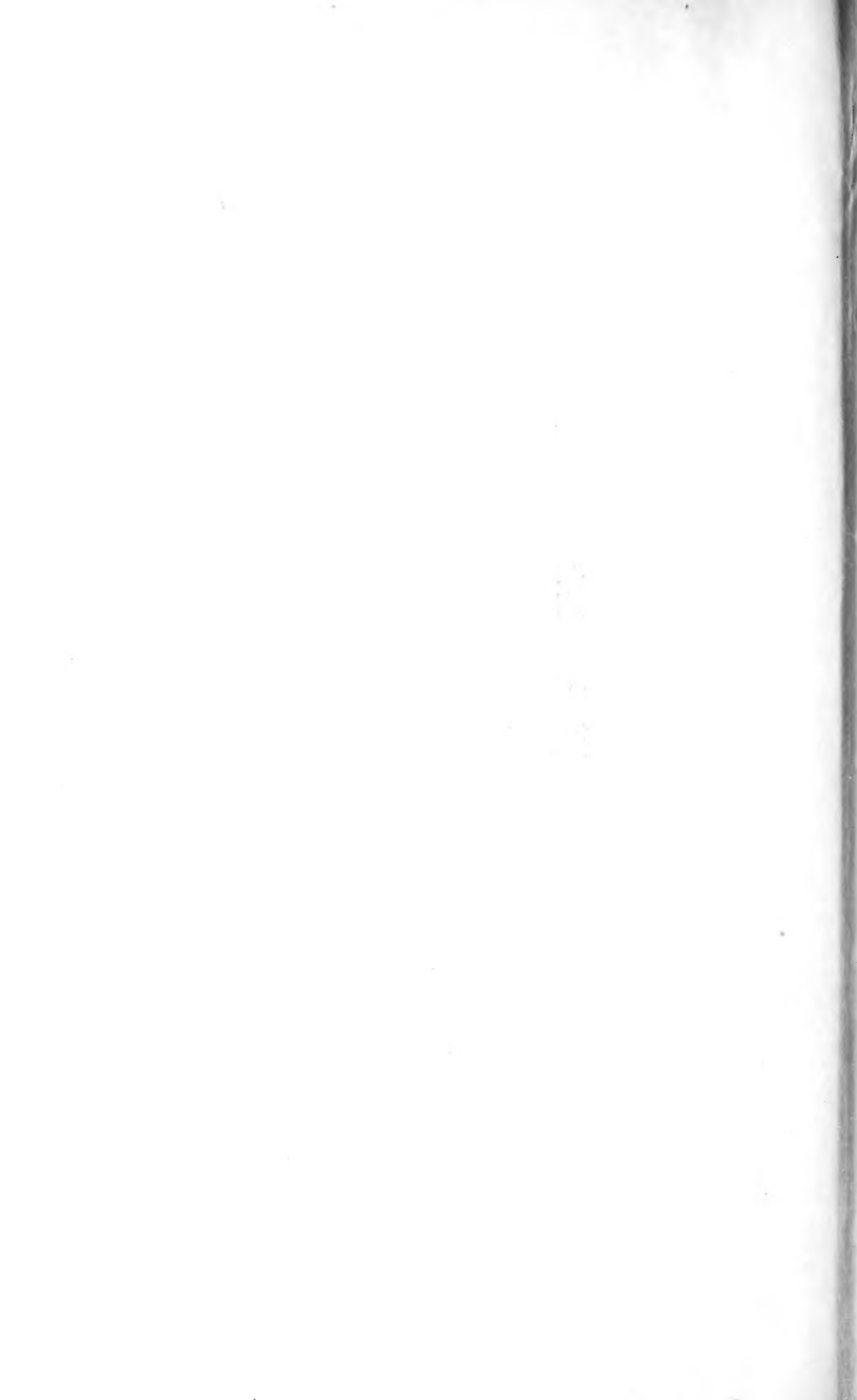
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